Statistical analysis of neuronal growth: edge dynamics and the effect of a focused laser on growth cone motility

T Betz¹,², D Koch, B Stuhrmann, A Ehrlicher and J Käs
Institute of Soft Matter Physics, Linnéstraße 5, 04103 Leipzig, Germany
E-mail: timo.betz@curie.fr

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Abstract. The neuronal growth cone is a small dynamic structure at the tip of neuronal extensions that guides each neurite extension to its correct partner cell. To reach the designated target, the growth cone integrates chemical signals with high accuracy and reliability. This signal detection operates close to the thermal noise limit and is, therefore of high interest not only to understand neuronal growth, but also to investigate the biological mechanisms of signalling and information processing under the influence of noise. To further investigate neuronal growth, a focused laser positioned at the leading edge of the growth cone is used to bias growth direction, however, the mechanisms of this influence are still unclear. We present a detailed measurement and analysis of the leading edge dynamics of laser treated and control growth cones. Based on the edge motility measurements, we can consistently describe neuronal growth with a stochastic model that allows a bistable potential and the noise intensity of the stochastic process to be extracted. The investigation of control growth cones that were not influenced by the laser reveals a nonlinear dependence of the noise on the overall activity of the growth cones. The presented analysis further quantifies the edge dynamics in growth cones that are manipulated by a laser. Growth cones that actively follow the laser show a tilt of the bistable potential in the direction of the laser to favour protrusions, but no significant changes in the leading edge growth velocity. This is in contrast to the potential changes observed in stationary

¹ Present address: Institute Curie, 11 Rue Pierre et Marie Curie, 75248 Paris, France.
² Author to whom any correspondence should be addressed.
growth cones that were influenced by the laser. Here, the laser does not tilt the potential shape, but increases the edge velocities, probably by an increase in actin polymerization velocity. These measurements provide new quantitative insight into the dynamics underlying growth cone protrusion and movement.

1. Introduction

Neuronal growth is a fundamental process during development and nerve regeneration. It is responsible for the correct wiring of the central nervous system, and is, therefore the base for every higher neuronal activity such as learning, communication and consciousness (Chilton 2006). To connect to a specific partner cell, a neuron grows long extensions, generally termed neurites. Later, each of these neurites becomes either an axon, which sends information away from the main cell body towards other partner cells, or a dendrite, which receives information from other cells and transmits it to the soma. Responsible for the growth process is a cone-like structure at the tip of every neurite, called the growth cone, manoeuvring the neurite through the organism by a continuous remodelling of its internal cytoskeleton. The main dynamics can be observed at the periphery of the growth cone, which is called the lamellipodium, a flat lamellar structure filled with a dense network of the polymeric cytoskeleton protein actin (see figure 1). The lamellipodium itself is responsible for the movement and the correct path finding of each neurite (Gallo and Letourneau 2004). The actin network in the lamellipodium is polymerized at the leading edge of the lamellipodium, transported towards its central region by a process called retrograde flow and finally depolymerized in the central region (Lin et al 1996). Thus, a continuous material flow of actin is maintained. Besides the flat and sheet-like lamellipodium, neuronal growth cones show finger-like protrusions called filopodia that extend from the lamellipodium. Filopodia consist of bundled actin filaments which show dynamics similar to the actin network in the lamellipodium (Mallavarapu and Mitchison 1999). In general, filopodia are thought to be important for probing the environment, whereas the lamellipodium is associated with the motility of the growth cone. It should be noted that the flatness of the lamellipodium as observed
in cell culture might be partially due to the flatness of the glass or plastic substrate that is usually used to culture the cells on. However, flat lamellipodia have been reported even under 3D in vivo conditions (Knobel et al 1999). Both lamellipodium and filopodia undergo drastic dynamical changes by switching randomly from protrusion to retraction states (Betz et al 2006; Mallavarapu and Mitchison 1999).

These dynamics are based on two processes within the lamellipodium. The protrusion of the lamellipodium edge is driven by the polymerization of new actin filaments right under the plasma membrane at the tip of the lamellipodium. This polymerization pushes the membrane forward and hence results in the observed outgrowth. The polymerization is tightly controlled by a set of actin associated proteins, responsible for filament capping, filament nucleation, monomer sequestering and depolymerization (Pollard et al 2000). However, this filament pushing is antagonized by the steady retrograde flow that contracts the newly formed actin network and transports it in a continuous motion towards the central part of the growth cone. This contraction is driven by myosin II motors (Medeiros et al 2006), and while transported away from the leading edge, the actin network steadily depolymerizes, thus providing monomers that can diffuse to the tip and repolymerize into the network. It is this retrograde flow that is responsible for the retraction of the edge. Fundamentally, the velocity of the leading edge is the sum of the retrograde flow velocity and the actin polymerization velocity. If the polymerization is faster than the retrograde flow, the edge effectively moves forward, but if the polymerization slows down it cannot compensate the retrograde flow anymore, and hence the edge retracts. Actin depolymerization is not required for the retraction of the leading edge, which is provided by the retrograde actin flow. Hence, the balance between polymerization and retrograde flow determines the growth behaviour. Manipulating either process results in a manipulation of the growth direction.

Furthermore, it is known that both the filopodia and the lamellipodium of the growth cone are involved in the correct detection of chemical cues that guide the growth cone to the correct partner cell. Previous investigations have shown that the signal detection mechanism is extremely accurate, working close to the thermal noise limit by allowing the growth cone to detect chemical gradients that are as small as only about one molecule difference across the diameter of the growth cone (Rosoff et al 2004). The question of how such accuracy and reliability is possible for a micrometre sized system immediately relates to an important field in biology and physics, which is the information processing of small scaled systems (Rosoff et al 2004). Within the volume of the growth cone, the number of signalling molecules is only in the order of $10^2$ ($\mu$M concentrations in femtolitre volumes), and the detection of small concentration differences furthermore requires to deal with a strong influence of thermal noise. Hence, an effective way of signal processing and detection should include the stochastic characteristics into the detection mechanism. Investigating the fundamental working principles of such stochastic signal detection relates to many biological questions like biological signal cascades, sensory systems and information processing in neuronal networks. But this subject is also of high interest for physicists that investigate small scaled systems, since at the micron scale Brownian motion becomes a dominant process that has to be considered in data analysis, interpretation and the design of experimental approaches. The controlled manipulation of the neuronal growth process is useful in the investigation of the biological processes that control growth cone motility and path finding.

It has been shown previously that a growth cone can be influenced by a focused laser which is positioned on the leading edge of the lamellipodium, an effect known as optical
guidance (Ehrlicher et al 2002, 2007; Stevenson et al 2006). The application of the laser results in two main morphological reactions of the growth cone, both associated with changes in the actin cytoskeleton (Ehrlicher et al 2002). First the laser induces a lamellipodium extension into the laser focus region. Furthermore, in actively translocating growth cones, the laser can change the direction of outgrowth, resulting in an optically guided turn. Up to now it is unknown to which extent these laser-induced changes of the lamellipodium dynamics can be attributed to changes in the stochastic fluctuations of the lamellipodial edge. Moreover, the lamellipodium dynamics differences between protruding and actively translocating growth cones had not been identified. To quantify the effect of the laser on the growth cone, we describe the growth cone’s lamellipodium dynamics in terms of a bistable stochastic process (Betz et al 2006), which is fundamentally different from the periodic lamellipodial edge oscillations observed in other motile cell types (Giannone et al 2004). This analysis method allows extracting the stochastic noise intensity and the shape of the bistable potential from the measured edge dynamics. On non-guided control growth cones, this analysis revealed a nonlinear dependence of the noise intensity on the growth cones’ activity. Thus, growth cones show signs of active noise that is controlled by the active dynamics of the lamellipodial cytoskeleton.

To quantify the effect of optical guidance, we analysed both stationary growth cones that showed pronounced lamellipodium extension into the laser and translocating growth cones that were guided by the laser. Fundamental differences in the extracted characteristics were found. When the growth cone actively moves into the direction of the laser, we detected a tilt of the bistable potential that is similar to the tilts observed in translocating control growth cones as reported previously (Betz et al 2006). However, in the case limited to lamellipodia extension into the laser, which was measured in stationary growth cones, this tilt was not detected, yet the laser did increase the edge velocity of the lamellipodium extension states, suggesting a faster actin polymerization due to the influence of the laser.

2. Material and methods

2.1. Cell culture and image acquisition

As introduced, the present work focuses on growth dynamics of the leading edge of neuronal growth cones. To quantify these dynamics, fluorescent and phase contrast time series of 10–20 min with a time resolution of 2–6 s have been recorded using a standard confocal microscope (TCS SP2 AOBS, Leica Microsystems, Bensheim, Germany) or a standard inverted microscope (DMIRB, Leica). All observations were done with NG108-15 (Hamprecht et al 1985) cells transfected with a pEGFP-actin vector (Clontech, Mountain View, CA) to fluorescently label the actin cytoskeleton. Transfection was performed using Nanofectin (PAA, Pasching, Austria) according to the product manual. Briefly, 5 |g of DNA and 16 |l of Nanofectin were each diluted in 250 |l of a 150 mM NaCl solution. Then both solutions were combined and incubated for 30 min before the mixed solution was added to the cell culture dish. In general, cells were cultured in Dulbecco’s Modified Eagle Medium (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (PAA, Pasching, Austria), 100 U ml⁻¹ penicillin/streptomycin (Sigma, St Louis, MO) and 10 mM HEPES (Sigma, St Louis, MO), and plated on laminin coated glass coverslips 24–48 h prior to observation. To ensure cell viability during the observation period, the Petri dish was placed in a sealed heating chamber to maintain pH stability and temperature. Furthermore, the objective was heated to 37 °C to prevent cooling.
of the samples. Image time series were recorded using an oil immersion objective (63× 1.4 NA or 63×, 1.25 NA, Ph 3, Leica Microsystems). To optimally detect GFP-actin in fluorescence imaging, the intensity of the excitation source was kept at a minimum which reduces bleaching. Generally, images between 40 and 60 µm wide were recorded with an image resolution of 1024 × 1024 pixels and a 4 times line averaging on the confocal microscope. On the inverted microscope, images were recorded with a resolution of 672 × 512 pixels.

2.2. Detecting the outline of a growth cone

The detection of the outline of a cell is a well known problem in the analysis of cellular motility (Stuhrmann et al 2005), but most classical image analysis methods are optimized for fast outline detection resulting in limitations regarding their resolution and reliability. To ensure precise and reliable outline detection a different method was developed that exploits a fit-function to determine the edge with subpixel resolution. This method is adapted from high resolution detection of single spherical beads (Thompson et al 2002), which is extended to allow edge detection of inhomogeneous systems like living cells and growth cones. The detection method was implemented in LabVIEW (National Instruments, Austin, TX) as described in the following. The edge detection algorithm works in a six step process: (i) image filtering, (ii) coarse edge detection, (iii) centre of mass (COM) detection, (iv) radial intensity profile extraction, (v) fitting procedure and (vi) reliability estimation of the detected edge position.

First, the images are filtered to remove detection noise in the images. In the next step, a threshold filter discriminates between pixels that are above, and below an automatically determined threshold level, resulting in a binary image. To gain a closed area that represents the coarse detected growth cone, particles that are disappearing during two step erosion are removed. For the further processing the uneroded images without the removed particles are used. Subsequently, the holes in all closed areas are filled and finally the biggest particle is chosen to represent the growth cone. To analyse phase contrast images, a more sophisticated coarse edge detection algorithm is used. This algorithm combines Prewitt filtering with extraction of high and low intensity regions. It should be noted that the intensity of phase contrast images has to be inverted to be consistent with the fluorescence images for the described fitting routine. The coarse edge detection is used to calculate a pixel based COM of the growth cone. Changes in the COM represent the total movement of the growth cone during the observation time. In the fourth step, 500 equally spaced radial line profiles as illustrated in figure 1, are extracted. (Note: the line profiles start at the outside of the growth cone, and point towards the centre.) The line profiles are centred on the coarse detected edge and include 1 µm inside and outside of the growth cone, ensuring that the real edge is covered in the line profile.

This line profile is the base for the fitting procedure that allows the edge position to be extracted with subpixel resolution. To infer a reasonable fit function, it is helpful to recall that the recorded fluorescence image can be regarded as a convolution of the exact edge with the microscope dependent point spread function (PSF), which represents the image of a single point-like particle. Generally, the PSF can be approximated by a Gaussian distribution (Sheppard and Shotton 1997), and the edge can be regarded as a Heaviside stepfunction $H(x)$. The convolution of any function $f(x)$ with a step function gives the integral of the function (Bracewell 1999):

$$f(x) \otimes H(x) = \int_{-\infty}^{\infty} f(x')H(x - x')dx' = \int_{-\infty}^{x} f(x')dx'$$

(1)
Figure 1. (A) Inverted confocal fluorescence images of a GFP-actin transfected neuronal NG108-15 growth cone. The edge position was determined from each image by extracting a line profile along 500 equidistant directions taken from the COM. To get subpixel position resolution, a Gaussian function was fitted to the derivative of each of these line profiles (C). The fit properties were used to determine the reliability of the detection. This is visualized in (A) by white dots for reliably detected and black for unreliably detected edge positions. The 95% confidence interval for the fit gives an error of <6 nm. The kymograph (B) shows the line profile as marked by the angle Θ in image (A), for the whole time series of 10 min. It allows to visualize how the edge moves outward and inward in a fluctuative way. The time series (D) shows a time evolution of a subpart of a growth cone, and exemplifies the dynamic movement of the lamellipodium and filopodia, (E) illustrates the subpixel resolution edge detection (blue), by comparing it to the best hand drawn contour (red). Hand drawn contours have a resolution limited by the pixel size (52 nm in the presented case), and show usually a low repeatability of several pixels, corresponding to several hundred nanometres.
where $\otimes$ denotes the convolution. Thus, taking the derivative, we can find:

$$f(x) = \frac{\text{d}}{\text{d}x} (f(x) \otimes H(x)).$$  \hspace{1cm} (2)

Since the measured line profile is the convolution of the Gaussian PSF with the step function, we regain the PSF by calculating the spatial derivative of the line profile and we determine the fit function to be a Gaussian distribution:

$$f_{\text{fit}}(r) = a \ast \exp \left( -\frac{(r - b)^2}{c^2} \right).$$  \hspace{1cm} (3)

Here, $a$, $b$ and $c$ are the three fit parameters that fully determine the Gaussian fit function. In this representation, $b$ represents the maximum of the Gauss function which is interpreted as the subpixel edge position. The fit was implemented using a Levenberg–Marquardt algorithm offered by LabVIEW. Using a fitting routine to detect the edge has several advantages. First, the whole shape of the line profile can be exploited to infer the edge, which results in the possibility of subpixel accurate edge detection. Using a 95% confidence interval, the error of a good fit is estimated to be $\pm 6$ nm. Furthermore, the fit allows inferring the reliability of the detected edge position. The edge detection was considered reliable if the fitted maximum was within the line profile, if $a > 0$ and if the variance of $b$ as provided by the fit algorithm was smaller than $0.7 \text{ pixel}^2$. These requirements turned out to be sufficient to separate reliably and unreliably detected edge values. Figure 1 illustrates the edge detection method and displays a sample growth cone with detected edge positions. Since 500 equidistant line profiles were extracted, the algorithm results in 500 COM edge distances for each image frame. However, in the presented work, the axonal stump was excluded from the analysis by only analysing the front half circle. In the figure, the reliably detected edge positions are marked by a white dot, whereas black dots represent unreliably detected edge positions. For further processing, all unreliably detected edge positions are replaced by an interpolation between the next reliably detected neighbours on both sides. As can be seen in figure 1, most unreliably detected edge positions are at the filopodia and at the neurite stump, both structures that are of no interest in this study. Thus, their effective removal from the data is convenient. Finally, the edge velocity is calculated by the change of the edge position within two subsequent image frames. For this, a common COM is to be calculated as the mean of the COMs of the both image frames used. The described procedure finally results in the edge velocity in 500 radial directions for the whole time series.

2.3. Optical guidance of neuronal growth

Optical guidance has been described previously (Ehrlicher et al 2002, 2007; Koch et al 2005). Briefly, a focused laser (800 nm) is positioned at the leading edge of the lamellipodium of a growth cone. To ensure that the laser interacts with the growth cone’s leading edge, the laser spot is repositioned depending on the movement of the lamellipodium. The laser position was adjusted using acousto optical deflectors (AA.DTS.XY.400@800 nm, AA Opto-Electronique, France) controlled by self written software implemented in LabVIEW. It has been reported that for optical guidance many different laser powers can be used, varying from 20 to 200 mW. To detect both, phase contrast and fluorescence images, a custom built shutter system was used to switch between phase contrast and epifluorescence. Phase contrast and/or fluorescence images were recorded every 5 s between each phase contrast or fluorescence image, respectively. The
Figure 2. Time evolution of the edge velocity of a given growth cone in a certain direction. The example illustrates the strong fluctuations of the edge speed. In further analysis, the edge velocity is separated into growth phases (velocity >0 $\mu$m min$^{-1}$, yellow) and retraction phases (velocity <0 $\mu$m min$^{-1}$, green). These phases are the base for further processing.

3. Results

3.1. Dynamical characteristics of the growth cone contour

The data for this study are the dynamical edge protrusion characteristics of neuronal growth cones. The velocity distribution and the residence time distribution (RTD) of growth and retraction phases were analysed. The unreliable detected edge velocities were corrected by interpolation using the reliably detected neighbours. To ignore any effects of the neurite stump, the analysis was restricted to the front half of the growth cone (0°–180°), where front is defined as the side opposite the stump. For the following histogram data representation the data from either the whole front part or certain angular areas (see figure 8(A)) are combined. During further processing, the time evolution of the edge speeds in each direction was separated into growth and retraction phases as exemplified in figure 2. The resulting phases are the base for further processing. First, the mean velocity distribution of these phases was investigated. To calculate this distribution, the mean speed of each phase that was at least two times longer than the sampling time (2–6 s) was determined and the particular speeds in any phase were replaced with the mean velocity of this phase. In the following, velocity will always denote the mean velocity of the separated phases. The first value of each phase has to be ignored since it is known that during the lag time between subsequent frame recordings a phase switch occurred and the value thus represents an overlay of protrusion and retraction movement, and its absolute value will be systematically too small. The resulting velocities are then represented in a velocity histogram.

Figure 3(A) presents a typical velocity histogram, showing a bimodal distribution with a peak for protrusion and retraction velocities at $-3.12 \pm 0.09$ and $3.19 \pm 0.07$ $\mu$m min$^{-1}$ (error representing 95% confidence interval of the Gaussian fit). The histogram was fitted with a
Figure 3. (A) The measured normalized velocity probability distribution (bars) of the growth cone in figure 1, which shows typical prominent protrusion and retraction velocities. To determine the peak position, a double Gaussian function was fitted to the histograms and the respective maxima where determined to be $-3.12 \pm 0.09 \mu \text{m min}^{-1}$ and $3.19 \pm 0.07 \mu \text{m min}^{-1}$ in the displayed fits. 

(B) Logarithmic plot of a typical RTD (bars), and an exponential fit function (red line).

double Gaussian distribution. For 44 measured growth cones, we calculate the average peak position to be $-1.49 \pm 0.53 \mu \text{m min}^{-1}$ for the retraction and $1.82 \pm 0.55 \mu \text{m min}^{-1}$ for the protrusion. Here, the error represents the standard deviation of the 44 measurements and already shows that the detected peak positions spread considerably. This spreading reflects the diversity of the recorded growth cones’ characteristics and the variability of the dynamics, since it does not depend on the experimental conditions (all data were taken with the same cell type and cell culture conditions).

To further illustrate the distribution of the peaks, figure 4 plots the retraction peak position over the extension peak position. Here each datapoint represents a single growth cone, and the position of the datapoints is defined by the most probable velocity as represented by the centre of the Gaussian fit that envelopes the retraction and protrusion velocity distribution. This plot illustrates that for many growth cones the absolute values of the most probable extension and retraction velocities are similar and thus they seem to be coupled to a certain extent. It is furthermore obvious that the data points accumulate at the upper right of the diagonal line. This means that in general the extension phases are similar or faster than the retractions, but never significantly slower. The clarity of the plot suggests a forbidden zone in phase space that prevents the peak of the retraction velocity to be at faster velocities than the peak of the extension phases. However, how these two processes are coupled to exhibit this unexpected behaviour is an open question. The finding of two peaks in the velocity histograms suggests that the two processes of extension and retraction are not correlated, but vary in an uncorrelated and probably stochastic way. The coupling that results in the observed forbidden zone might be due to different regulation mechanisms that control the different velocities, e.g. myosin activity for the retraction phases and actin polymerization for the extensions.

As mentioned before, the system exhibits strong fluctuations with stochastic characteristics. The stochastic nature of the processes is further supported by the analysis of the RTD.

The RTD is a probability measure of switching from one state to another within a given time. Thus, it is straightforward to determine the RTD from the data by counting how often the system stayed in a protrusion or a retraction state of a given duration. As presented in the logarithmic plot of figure 3(B) the RTD clearly follows an exponential decay, which is like a
Figure 4. Retraction peak velocity over extension peak velocity. Each data point represents a single growth cone. Equal peak velocities would be on the diagonal, and many data points do indeed arrange along this line. However, the plot shows a clear accumulation of points to the upper right of the diagonal. Thus, in about half of the measured growth cones the extension is faster than the retraction. Furthermore, we measure a great variety of peak values ranging from 0.5 to 3.2 µm min⁻¹.

Fingerprint for a Poisson process. Fitting an exponential function to the RTD yields values for the decay of the probability to find long growth or retraction processes. The following exponential function was used for the fit: \( p(t) = n \exp(-t \cdot r_M) \), where \( t \) is the actual residence time, \( n \) is a normalization constant and \( r_M \) is the measured decay rate. For the presented experiment, we get a decay rate for retraction processes of \( r_M^r = 0.108 \pm 0.004 \text{s}^{-1} \) and for the protrusion process of \( r_M^p = 0.095 \pm 0.002 \text{s}^{-1} \). Both are typical values for decay rates, and for the 44 growth cones investigated, we measure mean rates of \( \overline{r_M^r} = 0.143 \pm 0.075 \text{s}^{-1} \) and \( \overline{r_M^p} = 0.170 \pm 0.083 \text{s}^{-1} \). Thus, the typical decay time is on the order of 10 s.

Using this analysis it is now possible to quantify any inherent differences between images recorded with epifluorescence and phase contrast microscopy. For this, fluorescent and phase contrast images were taken in parallel with a lag time of 1 s. This means first a phase contrast image is recorded, and 1 s afterwards a fluorescent image is taken by changing from phase contrast to epifluorescence microscopy using a pair of shutters that control the illumination light. One shutter controls the fluorescence light and one the bright-field illuminations. The two
Figure 5. To evaluate the differences between fluorescence (A) and phase contrast (B) analysis, recordings of the same growth cone were compared. Two time series of fluorescence and phase contrast images were recorded in parallel and subsequently analysed using the introduced procedure. The resulting histograms are shown in green (fluorescence) and black (phase contrast). The differences are marginal, and the overall similarities are high. The scale bar is 10 µm.

Time series were then processed as described above. Sample images of fluorescence and phase contrast recordings and the resulting histograms are displayed in figure 5. The equivalence of both methods can be seen in the two plots for the velocity distribution and the RTD. In both cases, the histograms show high overlap, and the difference between the two recording methods can be used to estimate the error due to the reduced image quality of phase contrast recording as compared to fluorescence microscopy. The difference between the two histograms is described by the root mean squared deviation (RMSD) between the histograms that resulted from phase contrast and fluorescence microscopy. The RMSD is defined by:

$$\text{RMSD} = \sqrt{\frac{\sum (\sigma_{\text{fluo}} - \sigma_{\text{ph}})^2}{n}},$$

(4)

where $\sigma_{\text{fluo}}$ and $\sigma_{\text{ph}}$ are the frequencies of the bins in the respective histogram (fluorescence or phase contrast) and $n$ is the number of samples that are represented by the histogram. We measure an average RMSD of below 5% for 14 different histograms that compare fluorescence and the phase contrast time series.
3.2. Edge dynamics as a stochastic process

The exponential decay of the RTD together with the velocity distributions suggests modelling the time evolution with a stochastic description that includes active noise. The stochastic description has been briefly introduced previously (Betz et al. 2006) but will be presented in more detail in the following. The standard equation of motion that appreciates noise is a Langevin equation. However, in the presented analysis the observable is not the actual position of the edge but its velocity. Thus we have to switch to velocity space, and the Langevin equation now reads:

$$\frac{dv}{dt} = -\frac{dV(v)}{dv} + \sqrt{2\eta}\xi(t),$$

(5)

where $v$ is the edge velocity, $V(v)$ is a potential in velocity space, $\eta$ is the noise parameter of the system, and $\xi(t)$ represents Gaussian white noise with $\langle \xi(t) \rangle = 0$ and $\langle \xi(t)\xi(t+\tau) \rangle = \delta(t - t + \tau)$. To formulate the problem in velocity space instead of real space reflects the phenomenological finding that the edge velocity is stochastically fluctuating. Unfortunately, the noise term in the Langevin equation does not allow us to directly infer parameters like $V(v)$ or $\eta$ out of a time evolution. Furthermore, the presented data analysis yields the velocity histograms which simply represent the probability distribution of the velocities. Thus it is reasonable to switch to the Fokker–Planck equation that is equivalent to the presented Langevin equation. The Fokker–Planck equation can describe the measured probability distribution and reads as:

$$\frac{\partial p(v,t)}{\partial t} = \frac{\partial}{\partial v} \left( V(v)p(v,t) \right) + \eta \frac{\partial^2}{\partial v^2} p(v,t)$$

(6)

if we assume a constant noise parameter $\eta$. Here, $p(v,t)$ is the probability to find a certain velocity at a given time. For a neuronal growth cone, the overall behaviour does not change significantly during the 10–20 min recording time, and thus we assume for the probability distribution in steady state that $\frac{\partial p(v,t)}{\partial t} = 0$. The solution to this simplified equation is the Boltzmann distribution:

$$p(v) = N \exp(-V(v)/\eta),$$

(7)

where $N$ is a normalization constant. Thus, from the measured velocity distribution $p(v)$, we can derive the potential in terms of the noise, by taking the negative logarithm:

$$V(v)/\eta = \text{const} - \ln(p(v)),$$

(8)

where const $= \ln(N)$. It becomes obvious why the normalization constant $N$ is not important for the presented analysis, as it just reduces to an additive constant to the potential. With the presented calculation it is possible to infer how the potential is connected to the velocity distribution, but the potential is still scaled with the noise parameter that is not yet accessible. It should be noted that the potential as calculated in equation (8) is not a classical potential but is presented in velocity space. Thus, its derivative with respect to velocity would be equivalent to the reduced forces of the classical description. In other words, the potential simply gives the probability depth of the measured edge velocity.

So far, for all the calculations only the velocity histogram was used. However, it turns out that the RTD can be included in this analysis to finally gain the noise parameter $\eta$ and thus the scaling of the potential. Figure 6 shows the potential that results from the previously presented velocity histogram (figure 3) using equation (8). The potential shows two metastable minima ($\alpha$ and $\gamma$), separated by an energy barrier with the unstable peak at $\beta$ and represents a bistable
Figure 6. Calculated potential of the growth cone in figure 1 using (8). The arrows visualize the stochastic switching between a retraction (left) and a protrusion (right) state. $\alpha$ and $\gamma$ denote the potential minima, and $\beta$, the potential maximum. The potential can be used to infer the noise parameter for switching from retraction to protrusion and vice versa.

In terms of this potential, the protrusion and retraction phases represent phases in which the system is in the right or the left state, and the switching between the phases reflects a jump between the two minima. Such systems have been studied in depth more than fifty years ago by Kramers, who investigated the decay of bistable systems that are subject to noise (Kramers 1940). Kramers was able to relate the potential to the decay rate of the metastable potential dips and the so-called Kramers rate can be calculated to be:

$$r_K = \frac{\sqrt{V''(\alpha) | V''(\beta)}}{2\pi} \exp \left( -\frac{\Delta V}{\eta} \right).$$

Unfortunately, we only have access to $V/\eta$, and thus we cannot calculate the second derivatives. However, this is easily fixable by dividing equation (9) by the noise, yielding the reduced Kramers rate:

$$\frac{r_K}{\eta} = \frac{\sqrt{V''(\alpha) | V''(\beta)}}{2\pi \times \eta} \exp \left( -\frac{\Delta V}{\eta} \right) \tag{10}$$

$$= \frac{\sqrt{V''(\alpha) | V''(\beta)}}{2\pi} \exp \left( -\frac{\Delta V}{\eta} \right). \tag{11}$$

Since, we can simply use our measurements to gain $V''/\eta$ and the potential barrier $\Delta V/\eta$, we can calculate the two reduced Kramers rates $(r_K/\eta)$, for the retraction and $(r_K/\eta)_p$ for the protrusion. In practise, the negative logarithm of a double Gaussian function is fitted to the potential, and this fit gives the numbers required for the reduced Kramers rate. According to the theory the measured decay rates $r_M$ should be equal to the calculated Kramers rates $r_K$, and we can use this to identify the noise parameter $\eta$ by simply dividing the measured rate by the reduced Kramers rate:

$$\eta = \frac{r_M}{r_K/\eta}, \quad \eta_p = \frac{r_M}{r_K/\eta}_p \tag{12}$$
where $\eta_r$ is the noise for switching from a retraction to a protrusion state and $\eta_p$ is the noise for the backward switch. Simply, this model allows finding a noise parameter by connecting the velocity histogram with the measured RTD, and thus allows to consistently connect the two measurements.

### 3.3. Active noise in neuronal growth

The proposed method is used to infer the active noise in the lamellipodium fluctuation dynamics of neuronal growth cones. In our analysis, we first compared the two noise values ($\eta_r; \eta_p$) that are measured for each growth cone. In 36 of the 44 investigated growth cones these two noise parameters were equal within the error. However, among the 44 growth cones the magnitude of the measured noise values varied considerably. This is quantified in figure 7 where the mean of $\eta_r$ and $\eta_p$ for each of the 44 growth cones is presented. Apparently, the noise is highly variable between different growth cones. Within our sample of 44 growth cones, we found noise parameters that varied in almost two order of magnitudes, namely between 0.4 and 10.5 ($\mu$m min$^{-1}$)$^2$ s$^{-1}$. In classical, passive stochastic systems, thermal motion is the usual source of noise that results in the fluctuation of small particles. In fact, if the particle size and the dissipation are well known, the fluctuation of such a passive system can be used to measure its temperature (Peterman et al 2003). Apparently, the noise intensities measured in the neuronal growth cones’ leading edge dynamics differ between growth cones despite the fact that the measurements were all recorded at controlled temperature and special care was taken to ensure cell viability. To investigate to what extent the inferred noise intensity depends on certain growth cone characteristics, the measured noise intensity of the different growth cones is plotted over their activity (figure 7). Activity of the growth cone is a new quantity that we define here to reflect the activity of a growth cone’s edge movement as measured by the edge velocity. Since the histogram as presented in figure 3(A) represents the statistical evaluation of the edge velocity we exploited this plot to define activity. Our results show that the edge velocity switches between two most probable velocities, represented by the two peaks of the velocity histogram plot. Since the peaks are the characteristic measure for the velocity in the two phases, we define the distance between these peaks as the activity of the growth cone. This measure is more reliable than other possible definitions like the integral of the histogram, or the average velocities. In these cases the high velocity tails of the histogram would have a strong influence. However, many of the high velocity phases are due to measurement errors like wrong edge detection in a single frame, or filopodia that move laterally along the edge. These errors mainly affect the high velocity tails of the distribution. By defining activity as the distance between the peaks in the histogram, we avoid the influence of these errors, since only the central part of the distribution is used. This part still represents most data points.

The noise presented in figure 7 is defined by the mean of the two measured noise values as explained in equation (12). We find a strong nonlinear dependence of the noise intensity on the activity of the different growth cones. This suggests that the growth cones can actively control the noise intensity simply by changing their activity. The visualization allows us to connect the phenomenological observation of variable noise to a characteristic quantity describing a growth cone’s dynamic, providing insight to understand the mechanism that controls noise intensity. Furthermore, the presented plot suggests an analytical connection between the noise intensity and the activity of a growth cone. A linear fit is not adequate to describe that data (grey line), as shown by the low $r$-square values of 0.45. Furthermore, a quadratic fit is likewise bad
Figure 7. Plotting of the measured noise value over the activity of the growth cone. The activity is defined as the distance between the retraction and the protrusion velocity peak in the velocity histogram. The plot shows a strong dependence of the noise on the activity of the growth cone. To illustrate possible functional relation between noise and activity, several fits are presented. In grey are a linear ($Noise = a \times Activity$) and a quadratic fit ($Noise = a \times Activity^2$), using all data points for the fit. Both fits are of low quality, and the presented distribution of data points suggests separating the data into two branches, in the following called the upper and lower branch (upper and lower with respect to the quadratic fit of all data). If the data of each separate branch are fitted with a quadratic function (red and pink), a very good agreement between the quadratic function and the measurement can be achieved.

(grey line) although its $r$-square value is with 0.61 better than the linear fit function. The low $r$-square value of the quadratic fit is due to the scattering of the data. Hence, a phenomenological investigation suggests separating the data into two populations (called upper and lower) with different characteristic dependence between noise and activity. For the analysis the data points are split with respect to the quadratic fit. This separation allows very good quadratic fits of the presented data, with $r$-square values of 0.95 for the upper and 0.98 for the lower population. However, it should be noted that the splitting is based on a pure phenomenological analysis of the data, and we have as yet no model explaining the quadratic dependence. Nevertheless, the noise does strongly and nonlinearly depend on the overall activity of the growth cones investigated.

3.4. The edge characteristics of an optically guided growth cone

Besides revealing the noise intensity, the analysis also quantifies the potential and the RTD of the stochastic process that controls neuronal growth. For moving growth cones it has been reported that in the direction of growth, the potential that biases the stochastic process is tilted
Figure 8. Growth statistics of a growth cone that is guided by a focused laser. The time series (A) gives the change of the growth cone, and the green-cross defines the position of the laser spot. Images were recorded with a time lag of 5 s. (B) and (C) present the inferred potential and the RTD in the control direction and (D) and (E) in the direction of the laser. The shaded red and blue lines illustrate the changes in the potential dips in the different edge directions. Scale bar, 10 µm.

to favour protrusions (Betz et al 2006), and that this tilt eventually results in the final growth cone movement. Furthermore, it has been established that optical guidance can control the direction of a growing neuron. Therefore, we investigated the effect of the laser on the potential shape. Two main effects of optical guidance have been reported (Ehrlicher et al 2002). Firstly, a pure lamellipodium extension into the laser and secondly, a turning of the whole growth cone structure towards the laser. The pure lamellipodium extension mostly occurs in stationary growth cones, which do not or only hardly translocate but show the introduced lamellipodium dynamics. If the COM was changing less than 1 µm min\(^{-1}\) during the 10 min observation phase, it was considered stationary (see figure 8). In contrast, in actively moving growth cones the COM moves during the observation time, as shown in figure 9, and only in these translocating growth cones was the optical guidance able to control the direction of growth. Hence, the laser is not able to change a stationary growth cone to a translocating one, but it can bias the growth direction if a growth cone is actively growing.

The pure lamellipodium extension is best studied in a stationary growth cone that shows no net movement but does reorient towards the laser by increased lamellipodium extensions as presented in figure 8. To determine if the growth cone was stationary, the position of the COM was evaluated during the 10 min recording time. To separate the effect of the laser from the
natural edge dynamics, the analysis was done separately on two regions, one in the direction of the laser (red in figure 8) and one in the direction without the laser (blue in figure 8). Figure 8(A) shows a fluorescent time series of an analysed growth cone, where the laser was turned on at time 00 min. The green-cross marks the position of the laser. The laser itself is not visible since the applied fluorescence filters fully suppress any radiation at the wavelength of the laser (800 nm). For the presented experiment, we used a laser power of 80 mW at the growth cone. The position of the laser was visualized before and several times during the experiment by manually turning the fluorescence filters. To better show the effect of the laser on the lamellipodium extensions, the analysis of a stationary growth cone is presented. This growth cone did not significantly move but showed typical lamellipodia protrusions influenced by the applied laser. As presented in the time series of figure 8, the overall orientation of the growth cone changed from left to right. To investigate how the reorientation is accomplished and which properties were changed by the laser, the calculated potential and the RTD have been analysed. First, the characteristics in the control direction (figures 8(B) and (C)), are discussed. The control direction is marked in figure 8 by the blue shading (90–162°). The potential is shifted to favour retraction states, and the exponential decay of the RTD confirms this characteristic shift, since on average the retraction phases are slightly longer (decay rate \( r_{M,\text{control}} = 0.103 \pm 0.013 \text{ s}^{-1} \)) than the protrusion phases (decay rate \( r_{M,\text{control}} = 0.116 \pm 0.011 \text{ s}^{-1} \)). Due to the variance the difference is not statistically significant. However, it shows a clear trend that is consistent with the model. To measure the effect of the laser, these characteristics are now compared to the values in the direction of the laser, marked by the red shading in figure 8 (18–90°). The overall potential shape is significantly altered by the applied laser radiation. Regarding the retraction potential dip (left) the laser does slightly increase the retraction velocity, but shape changes of this branch of the potential are small. However, for the protrusion states (right), the potential is significantly wider (the standard deviation of the Gaussian fit is 1.5 \( \mu \text{m min}^{-1} \) in the direction of the laser and 1.1 \( \mu \text{m min}^{-1} \) in the control direction), and the minimum of the potential is increased from 1.66 \( \pm \) 0.14 \( \mu \text{m min}^{-1} \) in the control direction to 2.17 \( \pm \) 0.14 \( \mu \text{m min}^{-1} \) in the direction of the applied laser. Furthermore, both decay values of the RTD are smaller than in the control direction (\( r_{M,\text{Laser}} = 0.087 \pm 0.007 \text{ s}^{-1} \) for retractions and \( r_{M,\text{Laser}} = 0.108 \pm 0.011 \text{ s}^{-1} \) for protrusions). We conclude that the characteristic timescale of the protrusion and the retraction phases is increased if a laser is applied to the edge of a stationary growth cone. However, the overall characteristic that the retraction phases are slower and longer than the protrusion phase is still unchanged. Thus, the optically guided reorientation of the growth cone is mainly due to the increase in mean velocity and a higher variability of protrusion velocities during the extension phases. Since the extension is driven by actin polymerization (Zhou and Cohan 2001), the data suggest that the laser increases the average polymerization speed. Interestingly, the retraction velocities are also slightly increased in the region influenced by the laser.

Furthermore, the noise values were analysed. It turns out that the mean noise in the control direction (\( \eta_{\text{control}} = 1.23 \pm 0.31 (\mu \text{m min}^{-1})^2 \text{ s}^{-1} \)) is smaller than in the direction of the laser (\( \eta_{\text{Laser}} = 2.07 \pm 0.14 (\mu \text{m min}^{-1})^2 \text{ s}^{-1} \)). This increase is consistent with the predictions from the active noise as discussed in the previous section. Thus, the laser not only increases the average protrusion speed, it also increases the noise within the system. Since the myosin driven retraction phases are almost unchanged, it is possible to attribute the change in active noise to the laser induced increase of actin polymerization. However, whether the increase in noise is due to the measured higher protrusion speed or vice versa is still an open question.
Figure 9. Time series (phase contrast) and statistical analysis of an actively translocating growth cone guided by a focused laser. The potential shows a clear shift to favour protrusion states, and the decay value of the RTD is decreased in the direction of the laser. Scale bar, 10 µm.

In a second step of the analysis, we focused on different growth cones that were actively translocating during the observation period. On these growth cones, the effect of the laser can be observed by the change of growth direction, as shown in figure 9. The laser was applied with a power of 55 mW at the growth cone. Here, the phase contrast time series as shown in figure 9 was used to extract the edge dynamics. The black contour marks the outline of the growth cone, and the laser can be seen in the upper left edge of the growth cone. In the observation time, the growth cone reorients to the left due to the applied laser, and in contrast to the previously presented case, the growth cone moves forward during the whole 20 min of observation time. Again, the potential and the RTD were investigated in a control direction which has no laser applied (upper graphs, blue) and in the direction of the laser induced extension (lower graphs, red). Apparently, the changes in the potential are different to the stationary case. In both directions the potential dips for the overall protrusion and retraction velocities remained almost unchanged (retraction: $-2.44 \pm 0.12 \mu m \text{min}^{-1}$ in non laser direction changes to $-2.33 \pm 0.21 \mu m \text{min}^{-1}$ in the guidance direction; protrusion: $2.41 \pm 0.10 \mu m \text{min}^{-1}$ in non-laser direction changes to $2.56 \pm 0.11 \mu m \text{min}^{-1}$ in the guidance direction). The potential, however, becomes tilted to the right, resulting in a higher probability to grow into the direction of the laser, and the final outcome is the optical guidance of the growth cone. The decay values of the RTD decrease in the direction of the laser. Whereas the decay
rates for the control direction are $0.079 \pm 0.018 \text{ s}^{-1}$ for retraction states and $0.076 \pm 0.013 \text{ s}^{-1}$ for the protrusion, both values are reduced in the direction of the laser, namely $0.063 \pm 0.011 \text{ s}^{-1}$ for the retraction states and $0.061 \pm 0.010 \text{ s}^{-1}$ for the protrusion states. The noise values are the same for both directions ($1.47 \pm -0.55 (\mu \text{m min})^2 \text{s}^{-1}$ in the direction of the laser and $1.47 \pm -0.76 (\mu \text{m min})^2 \text{s}^{-1}$ in the untreated direction). Thus the laser tilts the potential to favour protrusion states in the direction of the laser, as well as to reduce retraction.

These results indicate that the effect of the laser on stationary and on translocating growth cones is different, with regard to the process that is influenced by the laser. Furthermore, it is not possible to use the laser to change a stationary growth cone into a translocating one by just focusing the laser at the leading edge. In the biological context this suggests that the stationary phases of growth cones are fundamentally different from the translocating ones, which is also confirmed by cytoskeleton stains comparing stationary and moving growth cones (Kalil et al 2000).

4. Discussion

It is known that the actin cytoskeleton in the lamellipodium of a neuronal growth cone determines the dynamics of its leading edge and that a focused laser can influence these dynamics. The molecular details of the growth process are still not fully understood, but it is well known that actin polymerization pushes the edge forward, thus being responsible for the protrusion process. The leading edge retraction, however, is due to a steady contractile movement of the actin cytoskeleton. This contractile motion transports the whole structure away from the leading edge towards the centre of the growth cone. Up to now, it is still not known how the numerous chemical guidance cues finally target these two antagonizing processes to result in the controlled growth cone movement. To learn more about this important biological process, we investigated the edge dynamics of neuronal growth cones. In this work, we have shown that a high resolution analysis of these edge dynamics gives insight into the fundamental mechanisms that determine the lamellipodial dynamics, and that finally result in lamellipodium extension into the laser and in growth cone turning.

Our results allow the leading edge dynamics to be described in terms of a bistable stochastic process that quantifies characteristic properties like the biasing potential and the stochastic noise intensity. In particular, the measured histograms of edge velocity and RTD can be described by the stochastic process. This means that the biological process integrates inherent stochastic fluctuations of the system into the detection mechanisms. To gain more information about the underlying processes, the stochastic model was used to extract the noise intensity that is active in each growth cone. The analysis yields that the noise is a characteristic property for each growth cone. The large variation in measured noise intensity suggests that the growth cone is able to control noise intensity, and that the intensity varies strongly with the activity of a growth cone. The analysis also reveals that the noise intensity depends nonlinearly on the activity of the growth cone. This allows us to speculate that the biological mechanism is regulating the noise intensity that effectively acts on the stochastic process. Such a regulation would, therefore suggest that the system uses a process similar to stochastic filtering to optimally detect the signal in a noisy environment.

Furthermore, the measured active noise suggests that a realistic model of the lamellipodium has to include active processes. Previously proposed active gel theories that describe actin polymer networks far from thermal equilibrium (Kruse et al 2004, Kruse et al 2005, Liverpool
and Marchetti 2003) might be good candidates for such a description. In general, biological systems like cells are not in thermal equilibrium, but due to the complexity of cells it is very challenging to quantify the activity and its influence on the overall cell behaviour. Our results indicate that describing neuronal growth by a stochastic process allows for a quantitative description of the noise dependence on the activity of the growth cone. We find a strong nonlinear dependence that can be fitted by a quadratic function, but further measurements are required to give a final exponent that provides best fit to the data. However, the general nonlinear dependence is quite surprising, and it suggests that the stochastic model could be extended by including multiplicative noise to gain insight into the relevant processes. The finding that the noise intensity varies nonlinearly with the activity will hopefully trigger further theoretical investigation in the field of active processes. Neuronal growth cones could then provide a model system to study the properties of active noise in living cellular systems, and would thus help to understand how living cells can ensure proper functioning of signalling and information procession despite the constant noise that is characteristic for such small scaled systems.

To investigate the effect of optical guidance on the stochastic edge fluctuations, we further used a laser as a tool to induce lamellipodium extensions and to optically control the direction of a growing neurite. This technique has the potential to allow for controlled experiments to test the biological significance of the introduced stochastic model. We analysed the edge dynamics of both stationary and moving growth cones when treated with the laser. In the pure lamellipodium extension experiment, the whole growth cone did not translocate but stayed stationary. In this case, the edge protrusion velocity was increased in the direction of the laser. The higher protrusion velocity indicates that the laser does increase the polymerization velocity at the leading edge, which is the process responsible for the lamellipodium advancement. However, how the laser can increase the actin polymerization is still not certain. It has been previously suggested that the optical forces can bias the diffusion of actin monomers or other bigger protein structures involved in actin polymerization. This bias might locally concentrate them in the region of the laser focus, thus resulting in a higher polymerization rate (Ehrlicher et al. 2002). Another possibility is that the laser pulls on the membrane, reducing the load on polymerizing actin filaments, thus favouring forward protrusion. Optical heating is another potential factor that one might consider, however, it has been shown that heating is unlikely to be a significant effect in optical neuronal guidance (Stevenson et al. 2006).

In contrast, when investigating growth cones that are actively translocating and that follow the applied laser during the time of observation, we record a tilted potential that favours protrusion, reduces retractions but does not exhibit faster edge velocity in the direction of the laser. This behaviour is similar to the changes observed in normal growing neurites, where the potential is tilted to favour the edge protrusion in a certain direction (Betz et al. 2006). Thus, we suggest that in a moving growth cone the laser induces a growth cone’s internal response that results in a translocation towards the laser. This implies that laser guidance might be an adequate tool to investigate the general mechanisms of growth cone guidance, helping to understand the biological processes that allow for the correct wiring of the neuronal network.

From a biological perspective these findings show that the effect of the laser differs between stationary and actively translocating growth cones. Whereas in stationary growth cones the laser changes the velocity of the edge it rather influences the distribution of growth and retraction phases for moving growth cones. This is a fundamentally different behaviour, since in the first case the effective speed is changed, which might be due to faster leading edge actin polymerization, whereas in the moving growth cones, the probability of longer growth phases is...
increased. In principle, both effects can lead to a change in direction, and based on these results, we suggest that in \textit{in vitro} growth cone guidance, different steering mechanisms might work at the same time. This can also explain how growth cones can respond simultaneously to different guidance cues, by assuming that each guidance cue controls different growth parameters like edge velocity, switching probability between the growth and retraction phases or the retraction speed.

In general, the description of neuronal growth by the introduced analytical model quantifies many different aspects of neuronal growth dynamics that finally determine the overall growth direction of the biological sample. This highly relevant system shows many features that are of great interest for the description of active processes, optical control of neuronal growth and for the physical description of growth cone motility and cell motility in general.

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