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ARTICLE

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In vitro analysis of neuron-glial cell interactions during cellular migration

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Abstract We used time-lapse microscopy to study the in vitro migration of neuronal cells from developing chick ciliary ganglion. These cells, when dissociated and cultured in a chemically defined medium, are able to migrate and to associate into clusters. We focused our attention on the study of the distribution of neuronal velocity components. Quantitative analysis of cell trajectories allowed us to demonstrate that, in many cells, velocities are well described by the Langevin equation, when deterministic components of the forces acting on the cells are taken into account. We also have shown that the majority of neurons whose movement is not purely random migrate in association with glial cells. We conclude that glial cells, by guiding neurons during migration, play an important role in the cell organization in vitro.

Keywords Ciliary ganglion · Cell locomotion · Langevin equation · Time-lapse microscopy

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Introduction

Cell migration plays a central role during embryonic and postnatal development of the nervous system. In both peripheral and central nervous systems, neuronal precursors and young neurons reach their final destination by migrating, with different modalities, along specific pathways (Hatten 1999; Perris 1997). Cell motion requires, in neurons as well as in the other cell types, the integration and coordination of several distinct processes, including morphological cell polarization, membrane extension, attachment and detachment to substratum, contractility and force generation (for a review, see Lauffenburger and Horwitz 1996).

An approach that has provided a large amount of information from in vitro and in vivo studies is time-lapse observations (Bradke and Dotti 1997; Gomez and Spitzer 1999; Lee et al. 1999). In addition, a statistical characterization of cell paths obtained from the observation of cell movement in vitro is a powerful tool to provide further insight into the process of motility. This approach has been successfully applied to the motion of different cell types (Stokes et al. 1991; Hartman et al. 1994; Palecek et al. 1997; Shenderov and Sheetz 1997; Maheshwari et al. 1999): it characterizes unambiguously cell motion parameters and provides a valid method for the comparison of the behaviour of different cells in response to environmental signals (e.g. growth factors and adhesion molecules).

Moreover, a quantitative analysis is required to test the validity of theoretical models and thus to obtain a more detailed knowledge of motility mechanisms and cell-cell interactions. Cell locomotion has been analyzed in the framework of the theory of Brownian motion by several authors (Dunn and Brown 1987; Stokes et al. 1991; Parkhust and Saltzman 1992; Schienbein and Gruler 1993) and, in particular, the Langevin equation has been used to formulate predictions of cell velocities. It has been suggested in the literature that the motion of the cells is not just

random, i.e. it may be affected by nonstochastic forces. This is, for example, the case of chemosensory movement: the directional bias in velocity due to the presence of a chemoattractant gradient is described by Stokes et al. (1991) by the addition of a deterministic drift function to the Langevin equation. In addition, a nonstochastic and periodic component of the force driving the motion has been proposed for both *Dictyostelium discoideum* and human neutrophils (Hartman et al. 1994; Shenderov and Sheetz 1997). In these cells the deterministic behaviour has been suggested to arise from an internal cellular motion program. In the literature, most of the analysis has been focused on second-order statistics, for instance by using autocorrelation of the cell velocity or mean squared displacement (Gail and Boone 1970; Parkhurst and Saltzman 1992; Dickinson and Tranquillo 1993; Shenderov and Sheetz 1997), whereas little attention has been devoted to the distribution of the components of cell velocity.

In this paper it will be shown that such distributions provide relevant information on cell dynamics; they allow us to test basic aspects of the Langevin description of cell motion and, in addition, to gauge, in a simple way, the effect of deterministic forces acting on cells. Theoretical predictions will be next compared with data on the velocities in the migration of a population of neural cells obtained from embryonic chick ciliary ganglion (CG). At the stage of development used in our experiments, E7, the CG is composed of post-mitotic neurons, a small percentage of neuronal precursors (Gilardino et al. 2000), satellite cells and undifferentiated glial precursors (Rohrer and Sommer 1983; Rudel and Rohrer 1992). All these cell types derive from the neural crest, a highly motile structure of the vertebrate embryo. CG cells retain the ability to migrate in vitro: when dissociated, uniformly plated and cultured in a chemically defined medium in the presence of basic fibroblast growth factor (bFGF), they are able to move and aggregate into clusters (Distasi et al. 1998). How cellular migration is regulated to give rise to such structures is still an open question; here, evidence will be provided of an unexpected role of glial cells in the organization of neuronal cell motion and nervous tissue.

The next section details the theory that supports the present study.

Theory

The motion of cells is, in general, brought about by stochastic forces, e.g. because of random interactions with the substrate or with the molecules of the medium; however, cell dynamics may also be affected by nonstochastic forces, due, for instance, to chemical gradients or interactions with other cells. Stochastic forces necessarily act on all cells, whereas deterministic forces may affect just some cells.

The more general way to describe formally the relation between these forces and cell motion is to use the Langevin equation that, in two dimensions, is, in components form:

$$\begin{aligned}\frac{\mathrm{d}v_x}{\mathrm{d}t} &= -\beta v_x + A_x^{\mathrm{r}} + A_x^{\mathrm{d}} \\ \frac{\mathrm{d}v_y}{\mathrm{d}t} &= -\beta v_y + A_y^{\mathrm{r}} + A_y^{\mathrm{d}}\end{aligned} \tag{1}$$

(Risken 1989), where β is the friction constant and $A^{\mathrm{r}} = \left(A_x^{\mathrm{r}}, A_y^{rmr}\right)$ denotes a random acceleration, which is generated by random forces, whereas $A^{\mathrm{d}} = \left(A_x^{\mathrm{d}}, A_y^{\mathrm{d}}\right)$ is the acceleration due to deterministic forces. In addition, $A^{\mathrm{r}}{}_x$, $A^{\mathrm{r}}{}_y$ are assumed to be generated by a Langevin force, i.e. they are normally distributed with zero mean and equal variance q, and:

$$\left\langle A_x(t_1) A_y(t_2) \right\rangle = q\delta_{xy}\delta(t_1 - t_2) \tag{2}$$

(see, for instance, Risken 1989).

From Eqs. (1) and (2) it is obvious that the components $v_x(t)$ and $v_y(t)$ can be analyzed independently and that results obtained for $v_x(t)$ hold also for $v_y(t)$; in other words, the results for the one-dimensional Langevin equation apply in this case.

The formal solution (Doob 1942) of the first part of Eq. (1) is:

$$\begin{aligned}v_x(t) = v_x(0)\exp(-\beta t) + \int_0^t A_x^{\mathrm{d}}(t')\exp[\beta(t'-t)]\mathrm{d}t' \\ + \int_0^t A_x^{\mathrm{r}}(t')\exp[\beta(t'-t)]\mathrm{d}t'\end{aligned} \tag{3}$$

For future reference we define $k_x(t) = \int_0^t A_x^{\mathrm{d}}(t')\exp[\beta(t'-t)]\mathrm{d}t'$ and suppose that $\lim_{t\to\infty} k_x(t) = \kappa_x$, where κ_x is a constant. Denote by $\tilde{v}_x(t)$ the solution of Eq. (1) in the case of purely random motion, that is when $A^{\mathrm{d}}{}_x = 0$, obviously $v_x(t) = \tilde{v}_x(t) + k_x(t)$.

Both v_x and $\tilde{v}_x$ are random variables: for a given initial velocity $v_x(0)$ the probability density functions (p.d.f.) of v_x and $\tilde{v}_x$ can be computed via the Fokker-Planck equation (see, for instance, Bhattacharya and Waymire 1990). It should be noted that, when $t \to \infty$, the p.d.f.'s of the velocity component $\tilde{v}_x$ is:

$$\tilde{p}(\tilde{v}_x) = \frac{1}{\left(\pi q \beta^{-1}\right)^{1/2}} \exp\left[-\beta\tilde{v}^2/q\right] \tag{4}$$

i.e. $\tilde{p}(\tilde{v}_x)$ is a Gaussian p.d.f. with mean value $\langle\tilde{v}_x\rangle = 0$ and variance $\sigma^2(\tilde{v}_x) = (2\beta)^{-1}q$; moreover, $\sigma^2(\tilde{v}_x) = (2\beta)^{-1}q$ is an invariant probability density, i.e. it is independent of t.

Under the assumption that $\lim_{t\to\infty} k_x(t) = \kappa_x$, where κ_x is a constant, it is straightforward to show that the p.d.f. $p(v_x)$ of v_x is also an invariant Gaussian density function with $\langle v_x\rangle = \kappa_x$ and $\sigma^2(v_x) = \sigma^2(\tilde{v}_x)$. The invariance of $\tilde{p}$ is a consequence of the fact that the stochastic forces are of the Langevin type, whereas invariance of p requires, in addition, the limit of $k_x(t)$ for $t \to \infty$ to be a constant κ_x, which can be interpreted as the total impulse per unit mass weighted by the term $\exp\beta(t'-t)$.

The analysis carried out so far provides the basis for a set of predictions that can be tested experimentally: for cells moving only under the action of stochastic forces we should expect that, in the limit of t large, the component of the velocity along the x-axis be distributed according to an invariant Gaussian p.d.f. with $\langle \tilde{v}_x \rangle = 0$, whereas for cells affected also by nonstochastic forces the stationary distributions of the velocity components are guaranteed if κ_x, the weighted sum of all instantaneous deterministic accelerations, is a nonzero constant; in this case $\langle v_x \rangle = \kappa_x$. Of course, similar considerations hold for the y-axis.

The invariance of $p(v_x)$ and $\tilde{p}(\tilde{v}_x)$ implies that, for large t, the processes described by the Langevin equation are, at least approximately, stationary in the strict sense (Doob 1953) and the usual ergodic theorems apply; in particular, this means that for any function of interest f, the ensemble average and the time average are equal: $\langle f \rangle = \bar{f}$.

For future use we define also an index J similar to the chemotropism index used to characterize cell paths in chemotaxis (Zigmond 1977). The net displacement d during a time interval T is $d = [(x(T) - x(0))^2 + (y(T) - y(0))^2]^{1/2}$, so that d is the modulus of a vector d with components $d_x = (x(T) - x(0))$, $d_y = (y(T) - y(0))$; note that $d_x = \int_0^T v_x(t)\mathrm{d}t = T\langle v_x \rangle$, $d_y = \int_0^T v_y(t)\mathrm{d}t = T\langle v_y \rangle$ from the definition of the time average of v_x, v_y and from the ergodic theorem. Therefore, $d = T(\langle v_x \rangle^2 + \langle v_y \rangle^2)$. Next, denote by l the length of the cell paths defined by $d = T(\langle v_x \rangle^2 + \langle v_y \rangle^2)$ (see, for instance, Do Carmo 1976), and finally let $J = d/l$. The index J measures the amount of directionality of the motion; if the cell moves along a fixed direction, $J = 1$, whereas if the motion is completely random, $J = 0$, since $d = 0$ (as can be seen by introducing $\tilde{v}_x$, $\tilde{v}_y$ in the definition of d), and $l \neq 0$. Furthermore the ratio d_y/d_x provides some indication of the overall direction of deterministic forces.

Materials and methods

Cell culture and cell purification

Cell culture methods have been described in detail in Distasi et al. (1998). Briefly, ciliary ganglia were dissected from E7/E8 chick embryos and dissociated enzymatically (0.06% trypsin in phosphate buffered saline, PBS, without calcium and magnesium for 5 min at 37 °C) and mechanically (with a Pasteur pipette reduced in diameter). Cells were re-suspended in N2 medium (Bottenstein 1983) with human recombinant bFGF (5 ng/mL, Amersham, UK) and plated in the middle area of 40 mm glass coverslips (Bioptechs, USA). Before plating the cells, the micro cover glasses were coated with poly-D-lysine (100 μg/mL) and laminin (LN, 2 μg/cm^2). Two hours after plating, neurons were clearly recognizable from non-neuronal cells, by their phase-bright, rounded cell bodies and short processes. Immunofluorescence and electrophysiological techniques have demonstrated that birefringent, round cells indeed are neurons (Distasi et al. 1998; Gilardino et al. 2000).

Purified neuronal cultures were obtained, when required, by adopting a technique which depends on differences in sedimentation rates of cells in a liquid medium (Davies 1995). Sedimentation was carried out in a 25 mm cylindrical, siliconized, glass dropping funnel with a round glass outlet tap. The funnel was filled with 22 mL of DMEM-F12 plus 10% heat-inactivated horse serum, placed on a vibration-free surface in the cold (4 ± 1 °C) and left for 5 h. Dissociated cells were re-suspended in 2 mL of PBS (with calcium and magnesium) and carefully layered on the medium. After 3 h the cells were extracted in 6 mL aliquots into sterile tubes. The aliquots were examined with phase-contrast microscopy to determine what fraction was the most suitable for plating as described above.

In order to evaluate the influences of glial-derived soluble factors on neuronal motion, we carried out a control experiment: 20 h after plating the N2 medium with bFGF was replaced by medium collected from an enriched culture of glial cells.

If not otherwise specified, all chemicals were purchased from Sigma, USA.

Motility assay

Cell velocity components were measured using time-lapse videomicroscopy. At 20 h post-seeding, a micro cover glass was placed in a heated (37° C) live cell chamber system (Bioptechs, USA) on a Eclipse TE 200 inverted microscope (Nikon, Japan), equipped with a CCD video camera (PCO, Germany). Raw data were acquired every 20 s with an image acquisition board and software (IMAQ-Vision, LabVIEW, National Instruments, USA). The acquired images were then analysed using the GNU Image Manipulation Program and software subroutines written in the laboratory. The method to determine neuron's centroid was as follows. A threshold function was applied to each image, and a binary image was thus obtained, segmented in "particle" regions, corresponding to the body of neuron and a background region. Open contours were closed by applying a convex hull function (XITE, University of Oslo). For each particle we computed the centroid by averaging the x, y coordinate values. Note that the particle regions represent the neuronal soma, so neurite length changes do not influence the centroid motion. Single cell velocity components were calculated from the corresponding velocity vector. The latter was determined by dividing for each frame the cell centroid displacement vector by the time-lapse interval. Migration measurements were carried out over a period of about 9 h.

A time-lapse recording of the micrometric grid was carried out as a control for microscope drift; no changes in image position were found.

Results

The CG is composed of two main cell types: post-mitotic neurons and glial cells. At the starting time of observation, 20 h after plating, all neurons had extended neurites and were still sparsely scattered over the dish surface together with glial cells (Fig. 1a). After the time period of observation (about 9 h), most cells had moved from the initial position (Fig. 1b).

Cell motion occurred through two different mechanisms: cell body migration along neurites (Fig. 2a) and, more often, cell displacement in association with a glial cell (Fig. 2b). In addition, just a few neurons seemed to be still during the time of observation. In the temporal sequence shown in Fig. 2b, a glial cell carries a neuron toward another neuronal cell and, in turn, this complex joins other glial cells.

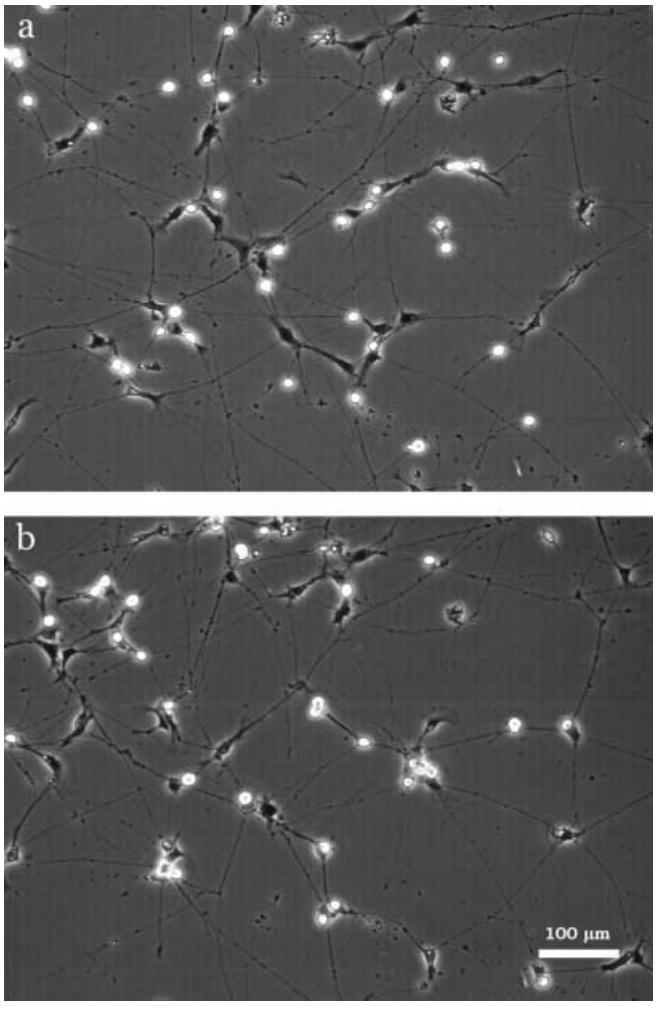


Fig. 1a, b Ciliary ganglion (CG) neurons' motility in the presence of glial cells. CG cells were obtained from a seven-day chick embryo after one day of culture. **a** Starting frame of an experiment in which neurons are cultured in presence of glial cells. **b** After 9 h, all cells have moved from their initial position

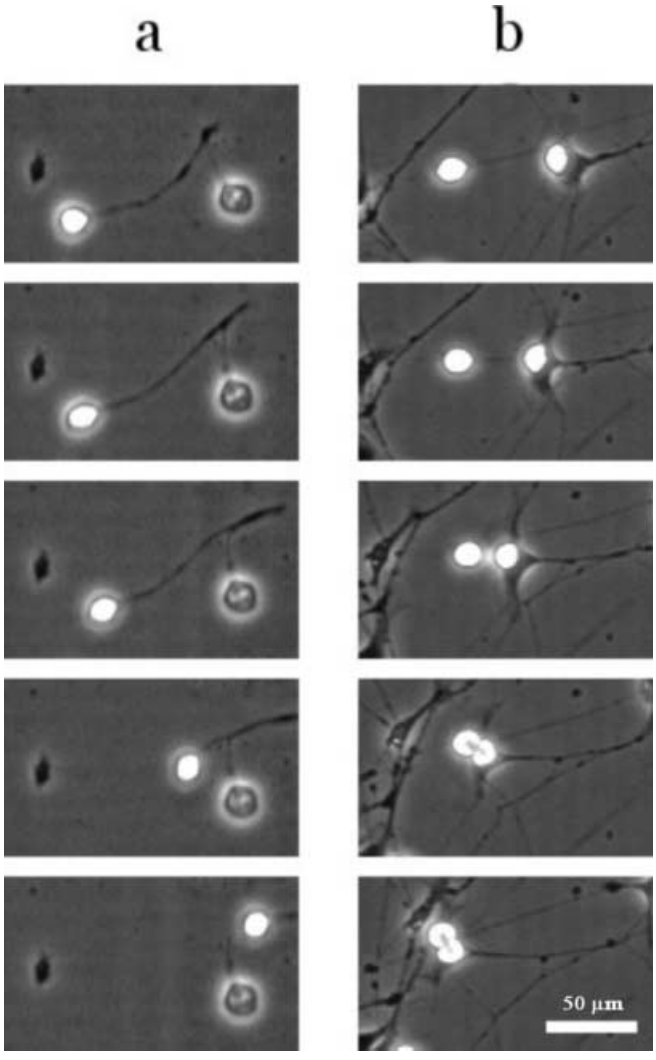


Fig. 2a, b Two different mechanisms of motion are observed in CG neurons. **a** *From top to bottom*, the sequence shows a neuron migrating along a neurite. **b** Neurons moving in association with glial cell

This behaviour strongly suggest an essential role for glia in the organization in vitro of neuronal tissue. To verify this hypothesis we cultured, in the same experimental conditions, purified neurons (see Materials and methods): we found that many cells, at the end of the observation time (9 h), were very close to their initial position (Fig. 3a, b).

In the Theory section it has been shown that, for large t, the distributions of the velocity components should be, at least approximately, invariant. Then if measurements of neuron velocity are taken after a relatively long time from the start of the process, the measured velocity components of a single neuron, denoted by u_x, u_y, must belong to the same invariant distribution, a Gaussian p.d.f., with mean values that may be different, depending on the types of forces affecting the motion of each neuron.

We have found that this is indeed the case; typical examples are shown in Fig. 4a, b, where the histograms of the velocity components of two cells are presented, together with the corresponding theoretical Gaussian p.d.f. From the values of the reduced χ^2, $\chi^2_v = 1.1$ ($P > 0.3$) for Fig. 2a and $\chi^2_v = 1.2$ ($P > 0.3$) for Fig. 2b, we can conclude that this is a reasonable fit (Bevington 1969).

This result is relevant from two points of view: on the one hand, it provides an empirical support of the model presented in the Theory section; on the other, it allows us to use the time series of each neuron to investigate the motility of a neural population, because the ergodic theorems ensure that, at least approximately, $\langle u_x \rangle = \bar{u}_x$ and $\sigma^2(u_x) = s^2(u_x)$, where $\bar{u}_x$ and $s^2(u_x)$ are the empirical mean and variance of u_x relative to a single cell, and similarly $\bar{u}_y$ and $s^2(u_y)$ denote mean and variance of the velocity components on the y-axis.

Probability density distributions shown in Fig. 4 differ in that the p.d.f. of Fig. 4a has a mean value $\bar{u}_x$ not

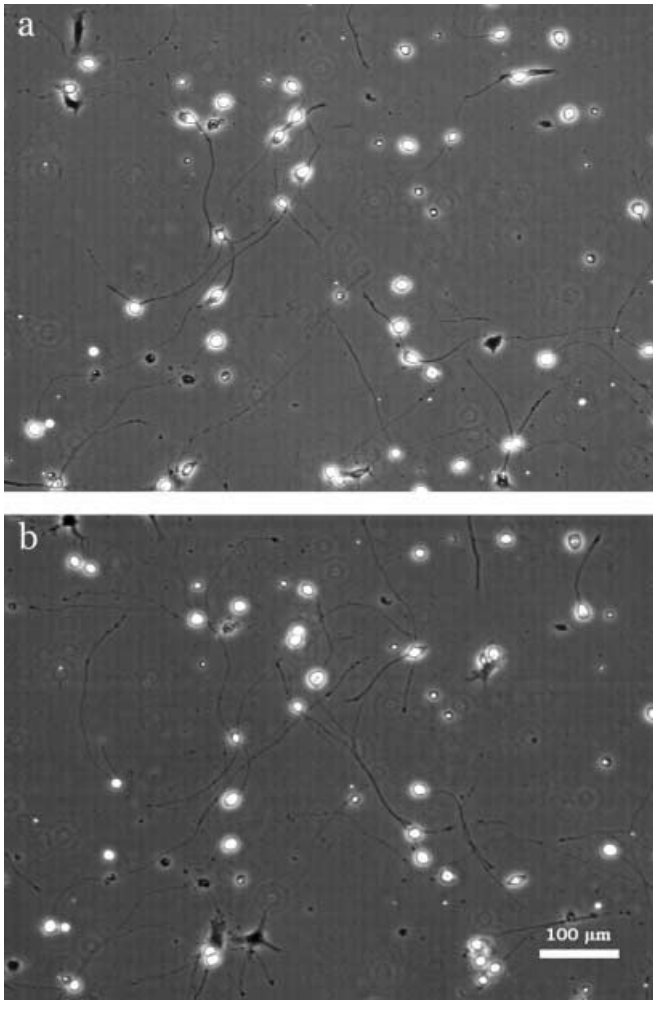


Fig. 3a, b Neuron motion in a purified culture. **a** Starting frame of an experiment with an enriched culture of neurons. **b** After 9 h, most neurons are close to their initial positions

significantly different from zero, whereas the opposite is true for the distribution of Fig. 4b; in general, our results show that the neurons can be classified, according to their velocity, into two classes: the first class is formed by neurons with both $\bar{u}_x$and $\bar{u}_y$ not significantly different from zero (*t*-test with $P < 0.01$; subsequently, neurons of this class will be called *R*-neurons), whereas in the second class belong neurons (called *D*-neurons) with at least one of $\bar{u}_x$, $\bar{u}_y$ significantly different from zero; out of 29 neurons, $n_R = 17$ are *R*-neurons and $n_D = 12$ are *D*-neurons. Mean values of the velocity components for *D*-neurons range from 0.005 to 0.08 μm/min, showing that, albeit different from zero, they are very small.

D-neurons could be further subdivided into two subclasses: neurons with just $\bar{u}_x$ or $\bar{u}_y$ different from zero and neurons with both $\bar{u}_x$ and $\bar{u}_y$ different from zero. We have chosen not to use this subdivision because we want, for greater generality, the classification to be invariant under transformations of the reference system and it is obvious that there exists always a rotation leading to a new coordinate system in which one of the components of the vector $\bar{u} = (\bar{u}_x, \bar{u}_y)$ is zero, whereas no transformation of coordinates can cancel both components.

The different behaviour of these two classes of neuronal velocities can be explained by considering the results of the Theory section: all neurons move under the action of a stochastic force, but for some of them, the *D*-neurons, the motion is affected also by nonstochastic forces whose resultant is different from zero and whose direction is not rapidly varying, at least with respect to the time of observation (9 h). Correspondingly, the motion of *R*-neurons is a simple random motion whereas for *D*-neurons the motion has a random component together with a deterministic component that gives rise to averages of u_x or u_y (or both) different from zero.

This hypothesis can be further tested by computing, for the neuronal trajectories, the index $J = d/l$ defined in

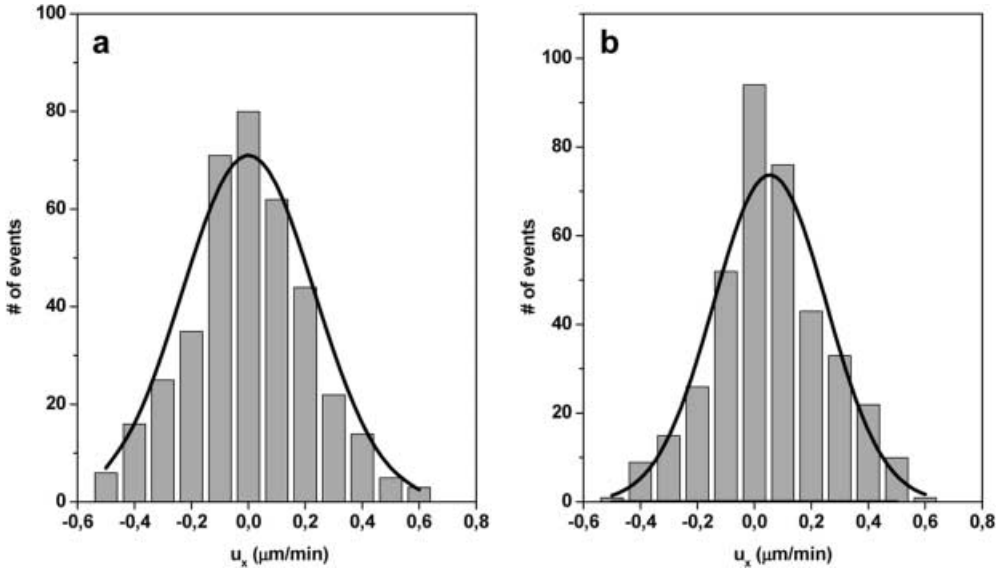


Fig. 4a, b Histograms of distributions of velocity components in two different cases. **a** Mean and standard deviation are $\bar{u}_x = 4 \times 10^{-5}$, $s(u_x) = 0.23$, and the mean is not significantly different from 0 (*t*-test, $v = 412$, $t = 0.004$, $P < 0.01$). The *continuous line* is the normal distribution with the same mean and standard deviations (χ^2_v, $v = 10$, $P > 0.3$). **b** $\bar{u}_x = 0.06$, $s(u_x) = 0.22$; in this case $\bar{u}_x$ is significantly different from zero (*t*-test, $v = 390$, $t = 5.5$, $P < 0.01$). The fit with the corresponding Gaussian gives $\chi_n u^2 = 1.2$, $v = 9$, $P > 0.3$

the Theory section. If the motion of *R*-neurons is random we must expect the corresponding value of J to take values very close to zero; on the other hand, if *D*-neurons move under the action of both random and deterministic forces, whose direction is not rapidly varying, larger values of J should be expected. In Fig. 5 are presented the trajectories of a *R*-neuron (Fig. 5a) and of a *D*-neuron (Fig. 5b), respectively; they show very clearly the different dynamics of the two classes.

The relation between the index J and the classes of velocity can be made more precise by a simple statistical analysis. Let J_R and J_D denote the values of J for class *R*- and *D*-neurons, respectively. Data for J_R and J_D from a sample of $n=29$ neurons ($n_R=17$, $n_D=12$) are represented in Fig. 6, which shows the frequencies of the values of J_R (filled circles) and J_D (open circles). It can be seen that there is a small overlap between the two distributions but data for J_R are distributed around low values of J, whereas values for J_D cluster around higher values. Averages are $\langle J_R\rangle = 0.05 \pm 0.01$ and $\langle J_D\rangle = 0.17 \pm 0.02$, and they are significantly different (*t*-test with $P < 0.01$); this finding is consistent with the hypothesis that a velocity component different from zero arises from a nonstochastic force, and that such a force does not vary too rapidly during the period in which the measurements are carried out.

In conclusion, experimental evidence points to the fact that neural motion is affected by Langevin forces and in addition the motion of some neurons (*D*-neurons) is also influenced by nonstochastic interactions. The question is now to verify that the source of such interactions is really the glial cells. To obtain this result, we have further subdivided the two classes of neurons according to the criterion of contact with the glial cells. In other words, we can assign to each neuron a further label: *C* if the neuron is in contact with glial cells or *I* if it is isolated: thus every neuron belongs to one of the four classes (*R*,*I*), (*R*,*C*), (*D*,*I*), (*D*,*C*). Table 1 shows frequencies of class membership for the sample of 29 neurons: from the table it is apparent that most *D*-neurons have moved in association with glial cells (75%). Conversely, most *R*-neurons have not had contact with glial cells (76%). A Pearson χ^2 test has been done to assess the non-uniformity of frequencies among classes; for one degree of freedom we obtained $\chi^2 = 7.53$, so we can reject the hypothesis of independence at the 0.01 level. This result shows a strong correlation between contact with glial cells and the deterministic component of the motion of *D*-neurons and hence suggests that the interaction with glial cells plays a central role in giving rise to the deterministic forces that act on neurons.

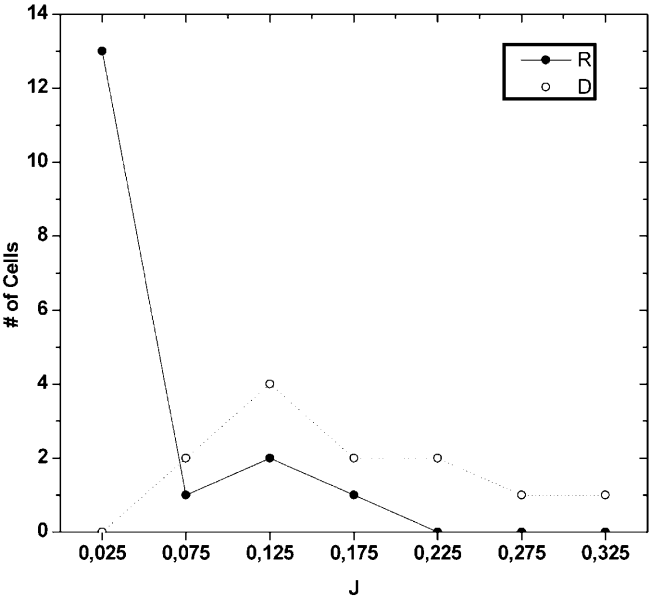
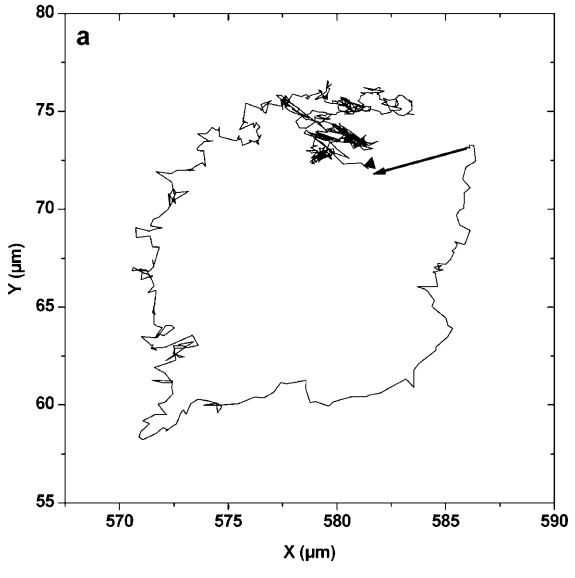


Fig. 6 Values of J plotted for the classes *R* and *D*. Each *point* is the number of cells whose index values are in interval $J \pm 0.025$. The *continuous lines* are added for visual clarity

Fig. 5a, b Typical trajectories of neurons. The *arrow* denotes the net displacement. **a** *R*-neuron ($J=0.02$). **b** *D*-neuron ($J=0.2$)



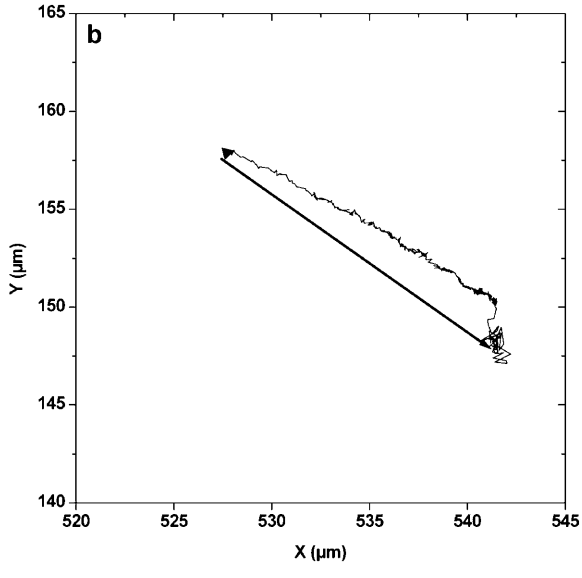


Table 1 Frequencies of class membership for classes (*R*,*I*), (*R*,*C*), (*D*,*I*), (*D*,*C*)

	R	*D*
I	13	3
C	4	9

As mentioned in the Materials and methods section, we have performed a control experiment to evaluate the influences of glial-derived soluble factors on neuronal motion. In this condition, neurons produce a network of fibers more developed than in the experiment carried out in N2 standard medium with bFGF. However, the data show no significant differences concerning the modalities of motion of neuronal cells: most neurons seem to be still, some move slowly along neurites and the largest neuronal displacement occurs when neurons move in contact with the few glial cells present in the field. The classification of *R* and *D* types of movement yielded the following results: eight (*R*,*I*) neurons, one (*D*,*I*), one (*R*,*C*) and two (*D*,*C*). Thus this experiment suggests that the neurite outgrowth enhancement by soluble glial factors per se does not affect neuron motility.

Discussion

The approach proposed here, based on first-order statistics of the components of the cell velocity, differs from most methods used to analyse cell motion, typically based on second-order moments of the modulus of the velocity (Shenderov and Sheetz 1997) or displacement (Stokes et al. 1991). These methods, while useful to study the stochastic components of cell motion, are not adequate to investigate the effects of deterministic forces. The main drawback is due to the small values of $\bar{u}_x$, $\bar{u}_y$ that make traditional methods insensitive to the existence of deterministic components of the motion. Consider, for instance, the autocorrelation of $\tilde{v}_x(t)$ and $v_x(t)$, denoted by $\tilde{R}(t_1,t_2)$ and $R(t_1,t_2)$, respectively. For t_1, t_2 large the autocorrelations depend only on $\tau=(t_1-t_2)$, as should be expected from the ergodic properties of the process; moreover, it is straightforward to show, by using standard formulae for $\tilde{R}$ and R (see Risken 1989), that as τ increases, $\tilde{R}(\tau)\rightarrow 0$ and $R(\tau)\rightarrow{\kappa_x}^2$. Since $\kappa_x=\bar{u}_x$ and $\kappa_y=\bar{u}_y$ are at most of the order of 10^{-1} μm/min and hence $R(\tau)\approx 10^{-2}$ (μm/min)^2 that, due to the fluctuations always present when dealing with experimental data, do not significantly differ from 0; in other words it is not possible to discriminate *D* cells from *R* cells.

Moreover, methods based on the modulus are insensitive to direction, whereas directionality is a characteristic of deterministic forces; in contrast, by using the components one can estimate, through the ratio d_y/d_x, or $\bar{u}_y/\bar{u}_x$, the overall direction of the resultant of such forces.

The empirical findings presented in the last section are in good agreement with the prediction of the model outlined in the Theory section; moreover, the two methods proposed to analyse the motion of neural cells, and in particular to discriminate between stochastic and deterministic components, work well and are consistent one with the other.

In addition, the relatively high number of cells belonging to classes (*D*,*C*) and (*R*,*I*) shows that the migration of CG neurons strongly depends on the physical contact with non-neuronal cells. This is a complex event that requires the exchange of signals for the reciprocal recognition and taxis and involves specific molecules in order to establish attachment and detachment to the substratum and the plasma membrane of the interacting cell (Rakic et al. 1994).

It is worth noting that the sensitivity of our methods allows us to analyse the very slow motion of cells like neurons and, therefore, it provides a means to study which factors (e.g. cellular adhesion molecules, growth factors, extracellular matrix molecules), together with their intracellular signal pathways, are involved in the regulation of the cell organization. In this perspective it can provide new insights into the dynamics of the repair mechanisms from damage induced by neurodegenerative diseases or by nerve injury.

The motion of the neuron-glial cell complex can be detected for most of the neurons examined in our experiments and it is predominant with respect to cell body translocation along neurites. The latter has been observed clearly in enriched neuronal cultures, an experimental condition which, obviously, strongly limits neuron-glia interaction. However, in this condition, most isolated neurons move slowly and randomly, without a well-defined motion along neurites. Body translocation along neurites could explain the behaviour of neurons that, according to our classification, belongs to the (*D*,*I*) class, whereas the dynamics of the (*R*,*C*) class can result from weak interactions between neurons and glial cells, whose effects are masked by the random interaction with the molecules of the surrounding medium and substratum. This behaviour has been observed even when cells were cultured in the presence of a conditioned medium obtained from an enriched culture of glial cells, an experimental condition that stimulates the neurite outgrowth.

Our observations recall some aspects of the glia guidance process found in the mammalian developing brain (Hatten 1999); it should be noted, however, that such a mechanism has never been described during the development of the peripheral nervous system. Several studies have pointed to the importance of adhesion molecules in guiding the migration of neural crest cells (see, for a review, Perris 2000), but little is known about the interactions between neuronal precursors and non-neuronal cells. Obviously, our results cannot be directly applied to the behaviour of neural crests in vivo; here we have studied the ability of a derivative of these cells to reorganize itself when isolated from the

embryo. Nevertheless, a similar role of glial precursors cannot be a priori excluded in the neural crest cell guidance or when these cells coalesce in the embryo to give rise to ganglia. In fact it is important to note that peripheral ganglion cells, even at relatively advanced stages of development, can be transplanted in early embryos and are able to reenter a migration pathway in the host (Le Douarin 1982). For these reasons, it will be interesting to further characterize the non-neuronal population of the CG, to clarify if it can be taken as a homogeneous population or if only a subset of cell is specialized in the regulation of neuronal migration and aggregation.

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