Gravitaxis in *Euglena*

12

Donat-P. Häder and Ruth Hemmersbach

Abstract

Motile microorganisms utilize a number of responses to external stimuli including light, temperature, chemicals as well as magnetic and electric fields. Gravity is a major clue to select a niche in their environment. Positive gravitaxis leads an organism down into the water column and negative gravitaxis brings it to the surface. In *Euglena* the precision of gravitaxis is regulated by an internal rhythm entrained by the daily light/dark cycle. This and the cooperation with phototaxis bring the cells into an optimal position in the water column. In the past a passive orientation based on a buoy mechanism has been proposed for *Euglena gracilis*, but now it has been proven that this flagellate possesses a physiological gravireceptor and an active orientation. Numerous experiments in space using satellites, rockets and shuttles as well as in parabolic flights have been conducted as well as in functional weightlessness (simulated microgravity) on ground-based facilities such as clinostats to characterize the gravitaxis of *Euglena*. The threshold for gravity perception was determined and physiological, biochemical and molecular components of the signal transduction chain have been identified. In contrast to higher plants, some algae and ciliates, *Euglena* does not possess sedimenting statoliths to detect the direction of the gravity vector of the Earth. The gravireceptors were found to be mechano-sensitive Ca^{2+}-conducting ion channels thought to be located at the front end of the cell underneath the trailing flagellum. When activated by gravity-induced pressure due to sedimentation of the whole cell body, they allow a passive influx of calcium along a previously established ion gradient. The entering calcium binds to a specific calmodulin (CaM.2) which in turn activates an adenylyl cyclase producing cAMP

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from ATP. This cAMP is believed to activate a specific protein kinase A (PK.4), which is postulated to phosphorylate proteins inside the flagellum resulting in a bending and thus a course correction and reorientation with respect to the direction of the gravity vector. The elements of the signal transduction chain have been characterized by inhibitors and by RNAi to prove their involvement in gravitaxis.

Keywords
Adenylyl cyclase • Calmodulin • cAMP • *Euglena* • Gravitaxis • Gravireceptor • Microgravity • Hypergravity • Protein kinase • Sensory transduction • Space flight

Abbreviations

DCFA-DAL	2′,7′ dichlorodihydrofluorescein diacetate
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
dsRNA	Double-stranded RNA
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
IBM-X	3-Isobutyl-1-methylxanthin
ISS	International Space Station
LED	Light emitting diode
mRNA	Messenger RNA
NiZeMi	Slow rotating centrifuge microscope
PAC	Photoactivated adenylyl cyclase
PCR	Polymerase chain reaction
PK	Protein kinase
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
RPM	Random Positioning Machine
rpm	Revolutions per minute
TEXUS	Technical experiments under microgravity
$TPMP^+$	Triphenylmethyl phosphonium ion
TRP	Transient receptor potential
UV	Ultraviolet radiation
W7	N-(6-Aminohexyl)-5-chlor-1-naphthalinsulfonamid

12.1 Introduction

Living organisms are exposed to a large number of environmental stimuli which are physical or chemical factors. Motile organisms such as bacteria, phytoplankton or animals respond to light (phototaxis) (Fraenkel and Gunn 1961; Häder 1991; Fiedler et al. 2005), chemicals (chemotaxis) (Clegg et al. 2003; Wadhams and Armitage 2004), the magnetic fields of the Earth (magnetotaxis) (Lohmann et al. 2004; Simmons et al. 2004), electrical fields or currents (galvanotaxis) (Votta and Jahn 1972) and temperature (thermotaxis) (Steidinger and Tangen 1996; Maree et al. 1999). All organisms on this planet are exposed to gravity. Most of them, including humans, have developed sensors to detect the direction of the gravitational field of the Earth and mechanisms to respond to the gravity vector (Hemmersbach et al. 1999a; Hock and Häder 2006; Ullrich and Häder 2006; Ullrich and Thiel 2012). The discipline of gravitational biology started in the nineteenth century when Julius Sachs (1882), Charles Darwin (1859) and Wilhelm Pfeffer (1904) studied the effects of gravity on plants (Hemmersbach et al. 2011) and demonstrated the role of the root cap in downward growing plants roots (positive gravitropism). They constructed machines (centrifuges and clinostats) in order to change the influence of gravity and observed the resulting gravity-related responses.

Viruses and bacteria are considered to be too small to react to the gravitational field; their swimming paths are mainly affected by Brownian

motion (Buttinoni et al. 2012). In contrast, unicellular phytoplankton and colonies clearly respond to gravity and use their responses for vertical migrations in order to optimize their spatial position in the water column (Richter et al. 2007). This has been studied in many motile microorganisms including *Tetraselmis*, *Dunaliella*, *Prorocentrum*, *Cryptomonas* and *Gymnodinium* (Rhiel et al. 1988; Eggersdorfer and Häder 1991a; Richter et al. 2007). The vertical migrations result in pronounced distributions of phytoplankton in the water column, the depth and density of which is affected by solar irradiance and the action of wind and waves (Piazena and Häder 1995; Häder 1997b; Raymont 2014). Vertical migrations of *Euglena deses* have been found also in the sand layer of the intertidal zone on the beaches in Sierra Leone (Taylor 1967). Their abundance is so high that they stain the sand green. This phenomenon is governed by diurnal migrations to the surface. Graviorientation and vertical migrations are also the basis for pattern formation in microbial populations (Bean 1984).

The phenomenon of gravitaxis has been studied for more than 100 years when cells were swimming in a vertical open or closed tube to the top (Platt 1899; Köhler 1921). The original term "geotaxis" was replaced by "gravitaxis" since this behavior is not solely induced by the Earth (greek gaia), but also by any heavy celestial body (e.g. Moon, Mars) and even by artificial accelerations such as on a centrifuge. The earlier investigations were basically descriptive observations by e.g. Jennings (1906) and Davenport (1908) which later on resulted in various interpretations and models how gravity is perceived. These earlier hypotheses have been reviewed by e.g. (Haupt 1962; Kuznicki 1968; Dryl 1974; Machemer and Bräucker 1992; Hemmersbach et al. 1999b). The interpretations were rather controversial. Stahl concluded from his observations that neither *Euglena* nor *Chlamydomonas* orient with respect to gravity (Stahl 1880). In contrast, Schwarz noted that *Euglena* swims upwards by an oriented movement and is not passively carried by currents (Schwarz 1884). He also concluded that this orientational movement is not induced by a higher oxygen concentration at the surface since the gravitational force of the Earth could be replaced by a centrifugal force. *Euglena* was capable of swimming against acceleration forces of up to 8.5 g. In contrast, Aderhold made an attraction to the higher oxygen tension at the surface responsible for upward movement in this organism (aerotaxis) (Aderhold 1888). A number of motile protists are especially useful as model systems including *Paramecium*, *Loxodes* and *Euglena* (Hemmersbach-Krause and Häder 1990; Hemmersbach-Krause et al. 1991b, 1994; Neugebauer et al. 1998; Streb et al. 2001) to study gravity-signal transduction chains within a single cell.

Also gliding organisms such as the desmid *Closterium* were found to show negative gravitaxis: when the cells were free to move on the surface of a glass plate within a water filled tube they were found to accumulate at the surface over night (Gerhardt 1913). Their meandering paths could be visualized by dying their slime trails. It was also noted rather early that light and gravity are competing stimuli in *Euglena*: in strong irradiances gravitaxis was weak while in the absence of light, gravity is the sole driver for upward movement (Wager 1911).

Movement in the direction of the center of the Earth guides the organisms into deeper layers of the water column; this is called positive gravitaxis (Fig. 12.1a). Moving away from the center is called negative gravitaxis and guides the organisms to the surface (Fig. 12.1b). Graviperception has probably been developed several times during evolution in different classes of organisms (Barlow 1995).

The precision of gravitactic orientation can be quantified using the r-value which runs between 0 (random orientation) and 1 (all cells swim in the same direction).

$$r = \frac{\sqrt{\left(\sum \sin\alpha\right)^2 + \left(\sum \cos\alpha\right)^2}}{n} \qquad (12.1)$$

where α is the deviation from the stimulus direction (here the gravity vector of the Earth) and n the number of recorded cell tracks (Batschelet 1981; Häder et al. 2005a). However, this value does not indicate in which direction the organisms swim. The mean swimming direction θ can be calculated as

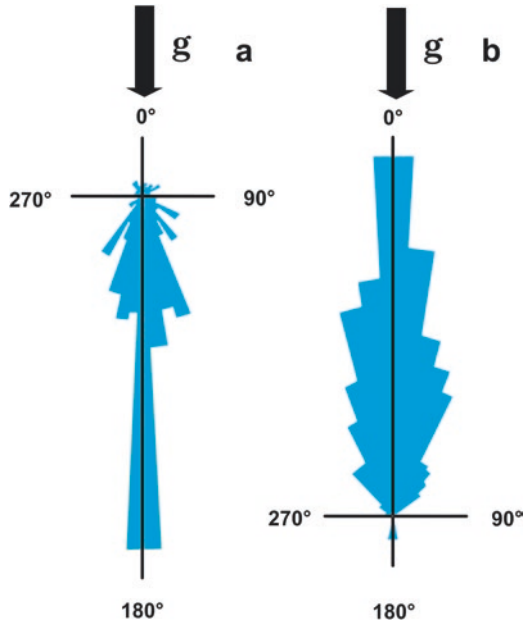

Fig. 12.1 (**a**) Positive gravitaxis in the ciliate *Loxodes striatus*. Tracks of swimming cells were recorded by automatic image analysis and the angle deviating from 0° (top of the swimming flask) were binned in 64 sectors. The circular histogram shows the percentage of tracks in each angular sector. Redrawn after (Hemmersbach and Häder 1999). (**b**) Negative gravitaxis in *E. gracilis* swimming in a vertical cuvette. Redrawn after (Häder and Vogel 1960)

$$\theta = \arctan\left(\frac{\Sigma \sin \alpha}{\Sigma \cos \alpha}\right) \qquad (12.2)$$

The direction of movement seems to be species-specific as in the case of ciliates (Massart 1891) and may depend on other environmental factors such as temperature as in the case of the flagellate *Chromulina*. Also in the ciliate *Paramecium,* the direction and precision of gravitaxis depends on temperature and the feeding status (Moore 1903) as well as the oxygen concentration in the medium (Hemmersbach-Krause et al. 1990, 1991a).

There have been many speculations on the mechanism of gravitactic orientation (Roberts 1970; Bean 1984; Barlow 1995). Some assume a pure passive phenomena, e.g. by buoyancy: when a cell is tail-heavy the tip points upwards which dictates the direction of movement (Fukui and Asai 1985; Grolig et al. 2004, 2006). In contrast to assuming that passive alignment in the water column results in vertical movements is the notion of an active gravireceptor driving a signal transduction chain which results in controlled steering movements orienting the cell parallel to the gravitational field of the Earth (Dennison and Shropshire 1984; Häder et al. 1995).

Since most cells are heavier than water their swimming velocity should be different whether they move upward or downward due to their sedimentation (Hemmersbach-Krause et al. 1993). Besides a pure vectorial addition or subtraction some species can even actively accelerate during upward swimming and decelerate during downward swimming, thereby at least partially compensate passive sedimentation (Häder et al. 2005a), a phenomenon which is called gravikinesis (Machemer et al. 1991; Machemer and Machemer-Röhnisch 1996; Gebauer et al. 1999; Häder 1999; Bhaskaran et al. 2009) and is found in many ciliates such as *Paramecium*, *Loxodes*, *Didinium* and *Stylonychia* (Krause et al. 2010) and is controversially discussed in *Euglena* (Machemer-Röhnisch et al. 1999; Häder et al. 2005a).

12.2 Ecological Consequences

Negative gravitaxis guides swimming organisms to the surface of the water column (Hemmersbach-Krause and Häder 1990). This is supported by positive phototaxis (Hopkins 1965; Häder 1988; Häder and Lebert 2001a, 2009). This behavior warrants that e.g. photosynthetic organisms accumulate close to the surface in order to harvest solar energy (Hopkins 1965). Often this movement pattern is modulated by a circadian rhythm; some flagellates start moving upward before dawn which results in their being near the surface when the sun rises (Ohata et al. 1997; Lebert et al. 1999a). However, since excessive irradiation can be detrimental these cells can reverse the direction of movement using positive gravitaxis which brings them to a lower level in the water column (Häder et al. 1999). This behavior can also be supported by negative phototaxis (Josef et al. 2005). In the presence of both light and gravity stimuli the cells respond to the resulting vector which depends on the incidence angle and irradiance of the actinic light (Kessler et al.

1992). Over the day the change from positive to negative gravitaxis and back results in vertical migrations within the water column (Eggersdorfer and Häder 1991b; Richter et al. 2007; Hu et al. 2014; Peacock and Kudela 2014). Dinoflagellates have been found to migrate up to 30 m (Yentsch et al. 1964; Taylor et al. 1966). When exposed to excessive solar radiation–especially high-energetic, short-wavelength ultraviolet radiation, some flagellates stop swimming actively and sediment passively (Häder and Liu 1990a; Häder et al. 1990a). Also in *Prorocentrum* exposure to solar or artificial ultraviolet radiation was found to impair graviorientation and velocity (Sebastian et al. 1994). In *E. gracilis* motility and gravitactic orientation are impaired by artificial and solar UV-B radiation (Häder and Liu 1990b).

Gravitaxis is also useful for non-photosynthetic organisms such as gametes and spores. Gametes have a higher chance of meeting their sexual partners when both use negative gravitaxis to reach the surface which enhances the gamete density. Zoospores usually swim around in their medium for a while which warrants their distribution in the habitat. After that they move to the bottom in order to anchor themselves and grow into a multicellular plant such as an algal thallus (Callow et al. 1997).

Some ciliates, such as *Paramecium* show negative gravitaxis (Hemmersbach-Krause and Häder 1990; Machemer and Bräucker 1992; Hemmersbach and Donath 1995). This behavior is modulated by the oxygen concentration in the water: at low oxygen tensions negative gravitaxis is less pronounced than at higher (Hemmersbach-Krause et al. 1991a) as shown in an experimental set-up without an oxygen gradient. In contrast, the ciliate *Loxodes* displays a strong positive gravitaxis at high oxygen concentrations, but is less well oriented at low oxygen concentrations. This behavior guides these microaerophilic ciliates into the sediment where they find their ecological niche (Fenchel and Finlay 1984, 1990; Finlay et al. 1993). This behavior demonstrates that under non-optimal conditions motile microorganisms swim upwards or downwards depending on their oxygen demands. After reaching an optimal area, swimming velocity is reduced and random swimming sets in.

Even not actively swimming microorganisms show vertical migrations in the water column. Cyanobacteria produce gas vacuoles to increase their buoyancy which brings them closer to the surface (Walsby 1987) where e.g. *Nodularia* ssp. are found to form large blooms (Sivonen et al. 1989). When exposed to excessive solar radiation they absorb the gas vacuoles and consequently sink out of the dangerous zone (Sinha et al. 2003). Diatoms produce oil droplets to alter their buoyancy which is also used to move up and down in the water column (Talbot and Bate 1987). However, using these examples of vertical migration may stress the term "gravitaxis".

12.3 Gravireceptors

12.3.1 Plants and Animals

Graviresponses have been observed in many motile and sessile organisms. Vertebrates possess otoliths in the inner ear, which have been found to press on the underlying cilia signaling the position of the animal with respect to the gravity vector (Rahmann et al. 1996; Anken 2006). Higher plants grow with respect to the gravity field of the Earth: primary roots grow downward (positive gravitropism) and stems upward (negative gravitropism). Thus, trees grow vertically up and not perpendicular to a slope of an ascending hill (Häder 2000). Lateral roots and branches often grow at a specific angle to the gravity vector (dia-or transversal gravitropism) (Willemoes et al. 1987; Kelly and Leopold 1992). The graviresponsive cells in the roots of higher plants are localized in a specific tissue in the root tip called statenchym located in the columella (Hou et al. 2004). However the growth response, guiding the root tip in the direction of the center of the Earth is located a few mm above the root tip which requires a signal transduction pathway between receptor and effector based on the transport of the plant hormone auxin guided by PIN proteins (Löfke 2011). The sedimentation of amyloplasts onto the endosplasmatic reticulum located in the lower part of the cell has been assumed to be responsible for the graviperception mechanism (Hensel and Sievers 1981). However, corn

mutants which lack amyloplasts in the root statenchym also show a (less precise) gravitropism; therefore the statolith hypothesis needs to be modified (Švegždienė et al. 2011). Mosses also seem to use sedimenting statoliths to sense the gravity vector (Salmi et al. 2011). However, it could also be possible that the contact between the amyloplasts and the membrane constitutes the stimulus and no force is necessary.

The freshwater alga *Chara* grows in shallow ponds where it is rooted in the sediment with rhizoids (Blancaflor 2013). In the tip of this unicellular structure there is an array of barium sulfate crystals which operate as statoliths (Sarafis 2013). When the rhizoid is turned into a horizontal position the statoliths are seen to sediment onto the lower surface and the rhizoid tip starts growing at the opposite side so that it bends until it is again in a vertical position (Braun and Limbach 2006).

In the unicellular ciliate *Loxodes* statoliths of barium sulfate have been identified within the so-called Müller organelles. A Müller organelle consists of a heavy body of barium sulfate fixed to a modified ciliary complex. Their function as mechanoreceptors (statocyst-like organelles) has been proposed and studied by Penard (1917), Fenchel and Finlay (1986), Hemmersbach et al. (1996, 1998) and Neugebauer et al. (1998). The specific density of the barium sulfate particles has been determined to be 4.4 g/mL (Hemmersbach et al. 1998). Severing the statoliths from their supporting cilia by laser beams abolishes gravitaxis in this organism. Electrophysiological studies indicated that this ciliate uses, in addition to the intracellular statocyst-like organelle, the mechanism of sensing its own cell mass via mechano-(gravi-) sensitive ion channels in the cell membrane to modify the membrane potential and thus ciliary activity to control the reorientation mechanism in the same way as in *Paramecium* (Nagel 1993).

12.3.2 Graviperception in *Euglena*

Earlier explanations for the orientation in the water column were based on a gravity-buoyancy model: If a cell is tail-heavy it would be oriented by buoyancy with the front end pointing upward (Richter et al. 2002a). Consequently, the trailing flagellum would propel the cell upwards. In a stable position the center of buoyancy is located above the center of gravity (Fukui and Asai 1985; Häder et al. 2005a). However, this hypothesis does not explain positive gravitaxis in young cultures. Microscopic analysis also does not show any heavy organelles, such as nucleus, chloroplast or mitochondria accumulated in the rear end of the cells. Kessler assumes that motile cells are passively reoriented by the torque generated by the swimming organism (Kessler and Hill 1997; Kessler 2007). If the gravity-buoyancy model would apply to the gravitactic orientation of *Euglena* one would expect that the cells smoothly reorient with respect to the gravity vector from a horizontal position. In fact, this behavior could be demonstrated by analysis of the reorientation maneuvers at high spatial and temporal resolution (Häder et al. 1997). The most pronounced directional change was found when the cells were oriented perpendicular to the gravivector of the Earth and is related to the calculated signal strength which follows the sine function of the angular deviation. In order to determine whether the cells are actually tail-heavy we compared dead (immobilized by injecting a cell suspension into liquid nitrogen) with living motile cells (Häder et al. 2005c). While the living cells showed negative gravitaxis with their front ends pointing upwards, the long axes of the immobilized cells pointed in random directions indicating that the cells were not tail-heavy; thus gravitaxis in *E. gracilis* cannot be described by a simple buoyancy model (Lebert and Häder 1999b).

Most of the analyses in this review on the graviorientation of *Euglena* and some other organisms were performed with a real time, computer-based cell tracking systems which we had developed in several iterations (Häder and Lebert 1985; Häder 1990; Vogel and Häder 1990; Häder and Vogel 1991; Häder 1992; Kühnel-Kratz and Häder 1993; Häder 1994b; Häder and Lebert 2000). A video camera with a microscope objective records the swimming tracks of numerous cells in parallel in a vertical cuvette in an infrared monitoring light, which does not affect

motility or photoorientation (Vogel and Häder 1990). The software calculates the size, form parameters, direction of movement and swimming velocity of the cells.

An alternative model for graviperception in cells with no obvious sedimenting statoliths was proposed by Roberts (1970) known as drag-gravity model. The gravity-buoyancy model is based on the assumption that one Reynolds number is sufficient to describe the sedimentation. The Reynolds number describes the ratio of inertial forces to viscous forces and it is dimensionless. Roberts introduced a second Reynolds number: one for the front and one for the rear end of a non-spherical asymmetrical body such as *Paramecium* or *Euglena,* which can be viewed as an assembly of two (or more) coupled spheres. Stokes's law predicts that a bigger (e.g. rear) end will sediment faster than the smaller front even when the specific density in the two parts is identical.

$$v = \frac{2(\rho_b - \rho_m) g r^2}{9\eta} \qquad (12.3)$$

where v = velocity, ρ_b = specific density of the body, ρ_m = specific density of the medium, g = acceleration (9.81 m s^{-2}), r = radius, η = viscosity of the medium [cP]. Experiments with immobilized *Paramecium* cells indicated that indeed the cells turned at a speed of 0.5–3°/s. The swimming speed of the cells should not have any effect on the rotational rate, however measurements in *Paramecium* showed such a dependence invalidating both the buoyancy-gravity and the drag-gravity model (Taneda and Miyata 1995). The same applies to *Euglena* where a change in the helical parameters was found during forward locomotion (Kamphuis 1999).

Another model for gravitactic orientation is the propulsion-gravity model which takes into account the helical path during forward locomotion (Winet and Jahn 1974). This results in the fact that the front end rotates with a larger radius that the rear end. This is due to the distance between the center of effort (the vectorial sum of all vertical forces) exerted by the flagella or cilia and the geometric center of the cell. Since the center of effort is closer to the front end of the cell than the geometric center, a torque occurs that moves the front end up and down in a horizontally swimming cell. However, this is not fully symmetrical since gravity applies an additional force. The sedimentation is countered by the viscosity of the medium especially at low Reynolds numbers and lifts the front end upwards. The vertical force of the flagellum (or cilia) is proportional to the sine function of the angular deviation of the cell from the vertical. During the upward part of the rotation it supports the sedimentation resistance. In the opposite position it counteracts the sedimentation resistance which finally results in an upward turning helical axis and finally a vertical reorientation of the cell. This model should work better in *Euglena* with a single flagellum at the front end than in *Paramecium* with cilia covering the whole cell body. However, a high-resolution analysis of the swimming paths did not show a strong correlation between the upswing of the helical movement and reorientation in *Euglena* (Kamphuis 1999).

So the basic question persists: What is the mechanism for gravitactic orientation in unicellular motile organisms such as *Euglena*? In this flagellate no obvious sedimenting particle(s) have been found. A possible alternative explanation could be that the whole cytoplasm with its organelles is heavier than water so that the cell content presses onto the lower cell envelope where it exerts a signal which could be used to control orientation movements (Sack 1997; Schnabl 2002). In fact, Wager had calculated the specific weight of a *Euglena* cell to be 1.016 g/mL (Wager 1911). In order to explore this possibility a simple experiment can help to distinguish between the action of an internal statolith and a heavy cell content (Fig. 12.2). In order to precisely calculate the intracellular specific weight *Euglena* cells were exposed to isopygnic centrifugation using layers with increasing Ficoll concentrations. The cells sedimented to the border between the layers with specific densities of 1.045 and 1.05 g/mL (Lebert et al. 1999b). The specific density of the cells depends on the culture age and conditions and varied between 1.046 and 1.054 g/mL (Lebert et al. 1999b). Freshly inoculated cultures showed

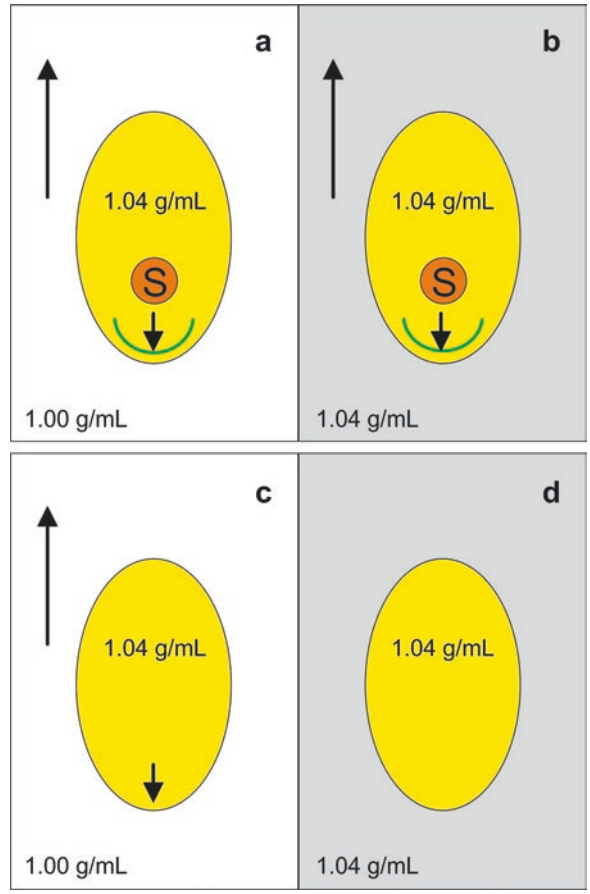

Fig. 12.2 Models for gravisensing. If an internal statolith plays the role of a gravireceptors the cell would be capable of orienting in a medium of a density of 1.00 g/cm^3 (**a**). It would also orient in a medium with a higher density (1.04 g/cm^3) (**b**), since it still presses on a receptive structure. If in contrast the cell content with an assumed specific density of 1.04 g/cm^3 presses onto a receptive structure, the cell would be able to orient in an outer medium of 1.00 g/cm^3 (**c**), but not in an adjusted medium with a specific density of 1.04 g/cm^3 (**d**), since the inner and outer medium are in an equilibrium

two bands during isopygnic centrifugation, indicating that the older cells were heavier than the young ones. One *Euglena* culture was kept for over 600 days completely enclosed. At the end of the experiment these cells had a specific density of 1.011 g/mL and did not show any gravitaxis (Häder et al. 2005a). In comparison, the cell bodies of the ciliates *Bursaria truncatella* and *Paramecium caudatum* have a specific density of 1.04 g mL (Krause 1999; Machemer-Röhnisch et al. 1999). In contrast, the barium sulfate statolith (Müller vesicle) has a specific density of 4.4 g mL (Hemmersbach et al. 1998).

The consequence of the hypothesis that the internal heavier cytoplasm presses onto the lower membrane to stimulate a gravitational signal transduction chain is that gravitaxis is abolished when the external and intracellular medium have the same specific weight and that the direction of movement should be reversed when the specific weight of the extracellular medium is higher than that of the intracellular space. This was in fact found by analyzing the swimming direction in Ficoll suspensions of increasing density (Fig. 12.3a–e) (Lebert and Häder 1996; Lebert et al. 1997).

A mathematical model for photo- and gravitactic orientation in *Euglena* by Hill and Häder describes a biased random walk (Hill and Häder 1997). It is based on the assumption that the cells initially swim in random directions, but when they deviate from the light direction or with respect to the gravivector they undergo a course correction. A similar strategy was found for e.g. chemotaxis of bacteria (Sourjik and Wingreen 2012).

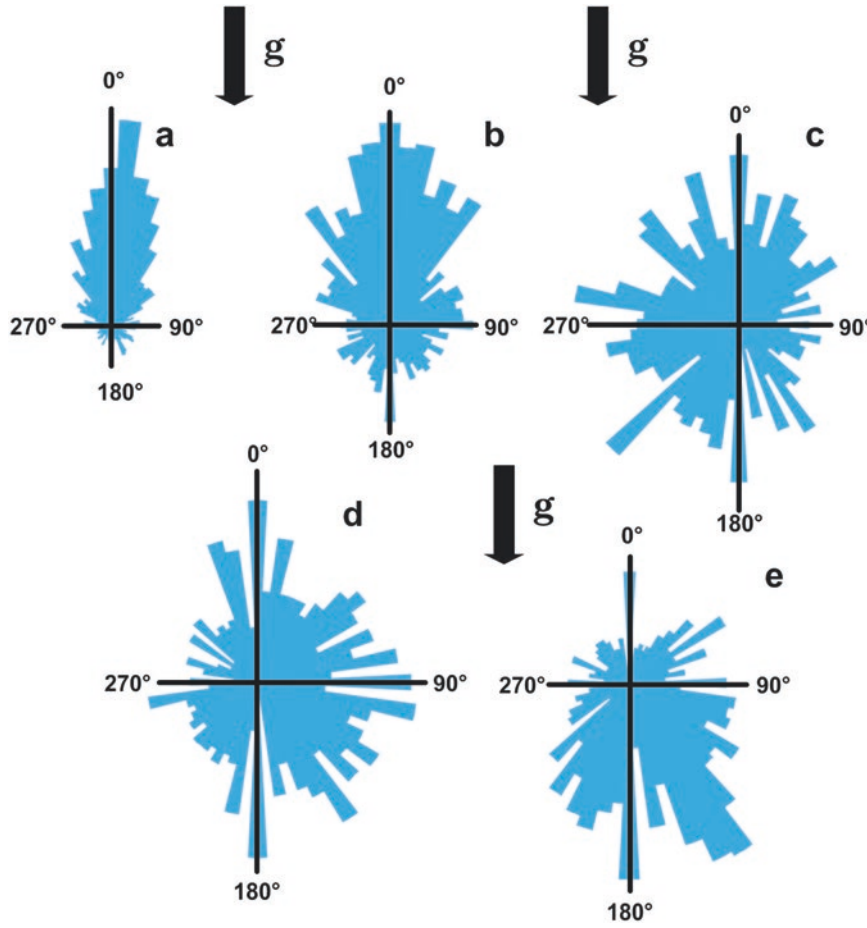

Fig. 12.3 Circular histograms of gravitactic orientation of *E. gracilis* in different Ficoll concentrations of 0% (**a**), 2.5% (**b**), 5% (**c**), 7.5% (**d**) and 10% (**e**). 0° indicates upwards; redrawn after (Lebert and Häder 1996)

12.4 Cell Behavior in Artificial Gravity: Simulated Microgravity and Hypergravity

In order to identify the mechanism by which an organism perceives an external clue resulting in an intensity-dependent response, the stimulus has to be modified and the impact on the response studied. In the case of gravity, representing a unique omnipresent stimulus, experimenters have to apply some tricks to alter its influence. Besides experiments in real microgravity which are rare to perform, different ground-based approaches have been developed based on various physical principles to simulate microgravity conditions on ground (for review see (Herranz et al. 2013; Brungs et al. 2016). Rotation of an object is applied in such a way that the influence of gravity is randomized. This approach is based on the assumption that biological systems need to be exposed to the gravity vector for a minimal presentation time in order to perceive it. It is postulated that under constant changes of the influence of gravity the object will lose its sense of direction and thus will show a behavior similar to the one seen under real microgravity conditions. So-called clinostats have either one single horizontal axis of rotation (2D clinostat) or two axes mounted perpendicular to each other (3D clinostat). The question arises how fast a

device has to be rotated to prevent an omnilateral gravistimulation but to achieve an optimal simulation of microgravity conditions for the exposed systems. Furthermore, the effective radius has to be taken into account, reducing on the one hand centrifugal forces which increase with distance from the center of rotation, but on the other hand providing enough space for e.g. undisturbed swimming of an organism such as *Euglena*. The effective centrifugal accelerations can be calculated by

$$a = \omega^2 r \qquad (12.4)$$

with r = radius, ω = angular velocity and a = acceleration. Based on studies such as the sedimentation behavior of immobilized cells, the speed of rotation was determined in the range of 50–100 revolutions per minute (rpm, fast-rotating clinostat) (Briegleb 1992; Herranz et al. 2013). In an experimental set-up for observation of *Euglena* or *Paramecium* in a clinostat equipped with a microscope the maximal centrifugal force of 3.5×10^{-3} g is calculated for the border of an observation field (radius 1 mm) and 4.5×10^{-2} g at the border of the observation chamber (radius 15 mm) during clinorotation at 50 rpm (Hemmersbach-Krause et al. 1993; Vogel et al. 1993).

Another approach to achieve simulated microgravity is to operate clinostats with two rotation axes, called 3-D clinostats, at a randomly changing speed and direction mode controlled by a dedicated algorithm. This device is called a Random Positioning Machine (RPM). Magnetic levitation is based on the principle that the gravitational force is counterbalanced by a magnetic force. A superconducting magnet with a high magnetic force field can levitate diamagnetic materials. It is assumed that a biological system experiences microgravity under this condition. *Euglena* has been exposed to the above mentioned simulation devices. All of them were equipped with in situ observation capabilities to study the impact on gravitaxis. Under two-dimensional fast clinorotation (60 rpm) the cells lost their gravitactic orientation and showed a random distribution comparable to the swimming pattern in real microgravity (Vogel et al. 1993).

Exposure on the RPM, however, indicated a completely different behavior. The random speed and random direction mode on the RPM induced numerous course corrections and even passive drifting of the *Euglena* cells, which demonstrates that *Euglena* experienced positive and negative acceleration forces (Herranz et al. 2013).

Exposure to high-gradient magnetic fields with different magnetic field strengths up to 30 T induced a perpendicular orientation of swimming *Euglena* with respect to the applied field, while *Paramecium* cells were aligned along the magnetic field lines in the same experimental set-up. This behavior clearly demonstrates that the magnetic field determines the orientation, preventing the organisms from random swimming known in real microgravity and furthermore demonstrated species-specific differences. *Euglena longa* (formerly *Astasia longa*) as well as the chloroplast-free *E. gracilis* mutant 1F showed no orientation perpendicular to the magnetic field lines. These results indicate that the chloroplasts in green *Euglena* operate as anisotropic structures which determine the orientation in the magnetic field. Thus, magnetic fields are not suitable to simulate microgravity for gravitactic unicellular organisms (Hemmersbach et al. 2014). Likewise, a centrifuge can be used to expose the swimming organisms to a higher acceleration (hypergravity) (Miller and Keller 2006). When this is combined with a video microscope the effect of hypergravity on the movement patterns of swimming cells can be studied (Friedrich et al. 1996; Häder 1996).

12.5 Real Microgravity in Space Experiments

Zero-g conditions are never completely reached on rockets, in a Shuttle or during parabolic airplane flights; the term microgravity is used to indicate low acceleration forces. During parabolic flights 10^{-2} g can be reached, on the International Space Station (ISS) 10^{-3} g and in drop towers 10^{-5} g (Schmidt 2004). The first real proof that upward orientation in an older *Euglena* culture is due to the detection of the gravity field of the Earth—and not to the magnetic field lines of the Earth or a

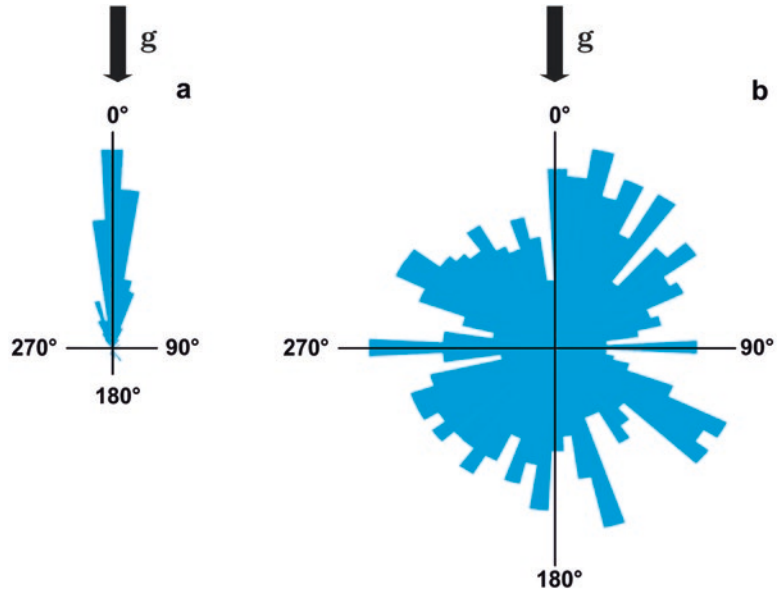

Fig. 12.4 On Earth at 1 g *Euglena* cells in their late logarithmic phase show a pronounced negative gravitaxis (**a**). In microgravity during a space flight on a TEXUS rocket they moved in random directions (**b**); redrawn after (Vogel et al. 1993)

chemical gradient such as oxygen—was provided by space experiments (Häder et al. 1990b). Cell cultures were flown on a TEXUS (technical experiments under microgravity) rocket launched in Kiruna (Sweden) on a parabolic path which provides microgravity for about 6 min (Häder et al. 2010). The swimming cells were observed online in space by means of a video downlink. Before launch the cells showed a precise negative gravitaxis (Fig. 12.4) and during the flight they moved in random directions (Vogel et al. 1993). Since the residual acceleration during the space flight is in the range of 10^{-3}–10^{-4} g the threshold for gravitaxis in *Euglena* must be higher.

Since the cells are heavier than the surrounding medium they sediment at the same time as they are swimming. This sedimentation velocity adds vectorially to the swimming velocity. Therefore the cells show a higher swimming speed in microgravity than at 1 g (negative gravitaxis) and an even higher speed during positive gravitaxis (Vogel et al. 1993)).

So what is the threshold for gravitaxis in this flagellate? The answer to this question was difficult to obtain since it required observing the cells in space (under microgravity) on a centrifuge with adjustable accelerations. This came true by the development of the NiZeMi (slow rotating centrifuge microscope) (Joop et al. 1989; Friedrich et al. 1996). This instrument was installed in the Space Shuttle Columbia for the second International Microgravity mission (IML-2) (Cogoli 1996; Häder 1996; Manieri et al. 1996). While in orbit under microgravity conditions, the cells were transferred into the NiZeMi and exposed to increasing accelerations in the range of 0–1.5 g and the swimming patterns were recorded for different accelerations. The results indicate that the threshold is found at ≤0.16 g, and that the precision of gravitaxis of *Euglena* was found to saturate at around 0.64 g (Fig. 12.5) (Häder et al. 1996). The dose-response curve shows a typical sigmoidal shape indicating the involvement of an active physiological receptor rather than a passive orientation in the water column, which would have been represented by a linear relationship (Häder 1997a). No adaptation to microgravity with respect to behavioral responses of *Euglena* was observed during the prolonged space mission as normal gravitactic behavior was found in populations returned to Earth. Analyzing the velocity distribution at different accelerations on the centrifuge indicated that this closely follows Stokes' law for sedimentation (Häder; 1994a; Häder et al. 1996). Thus *Euglena* does not show an adaptation in

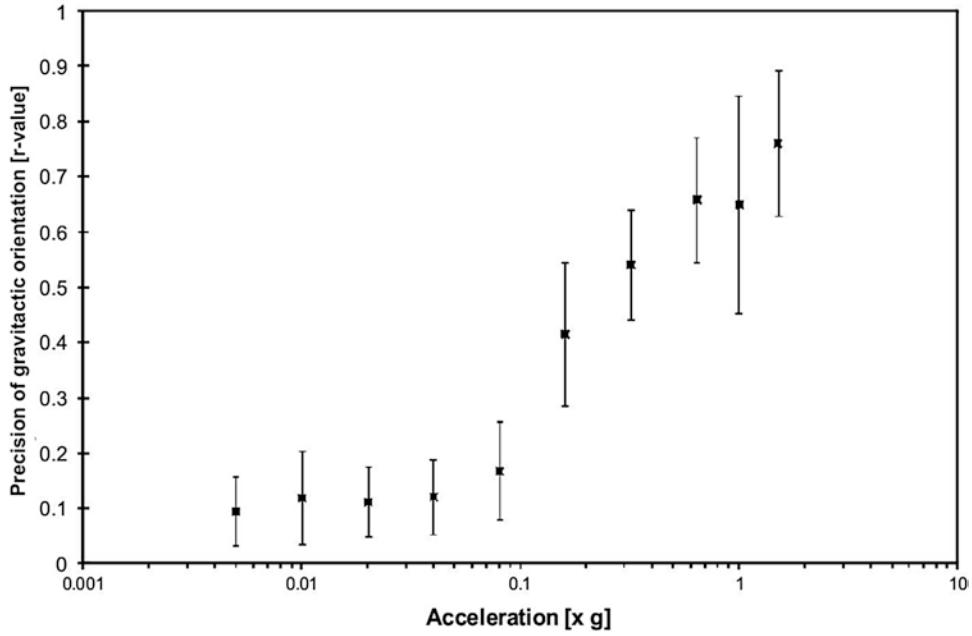

Fig. 12.5 Precision of orientation of *E. gracilis* exposed to increasing accelerations on the slow rotating centrifuge microscope on board the American Shuttle Columbia during the IML-2 mission. The threshold for gravitaxis has been calculated to be about 0.16 g from the pooled data from all four experimental runs; redrawn after (Häder et al. 1996)

swimming velocity (gravikinesis) which is in contrast to the ciliates *Paramecium* and *Loxodes* (Machemer et al. 1997; Machemer-Röhnisch et al. 1998). The gravitactic threshold could be further determined with more precision during a sounding rocket flight on a TEXUS rocket and was found to be below 0.12 g (Häder et al. 1997). Thus, the threshold for gravitaxis in *Euglena* would be sufficient for an orientation on Mars or the Moon (Kiss 2014). The threshold for graviperception in *Paramecium* was found between 0.16 and 0.3 g (Hemmersbach et al. 1996).

The next question is: what happens at higher accelerations (hypergravity)? In order to elucidate this problem the ground model of the centrifuge microscope NiZeMi was employed (Häder et al. 1991b). When the centrifuge is at rest the cells show a precise negative gravitaxis. At a total acceleration of 2 g (1 g Earth gravity plus 1 g from the acceleration perpendicular to the Earth gravitation field) the cells move along the resultant of the two forces. With increasing accelerations the cells orient more and more with respect to the centrifugal force up to 9 g. At higher accelerations (10 g) most of the cells were centrifuged down and could not swim against this force. Also *Paramecium* was found to be capable of swimming against an acceleration force of 8 g (Hemmersbach-Krause et al. 1992), while *Peridinium* could swim only against 3.8 g (Häder et al. 1991a).

As discussed above, the swimming speed results from the vectorial addition of the swimming velocity and the sedimentation velocity. The mean velocity decreased linearly with increasing accelerations of the centrifuge up to 9 g. In comparison, the sedimentation velocity of dead cells (killed with 0.1% glutaraldehyde) indicates a linear relationship of the sedimentation velocity with the acceleration force (Häder et al. 1991b).

12.6 Physiology of Gravitaxis in *Euglena*

12.6.1 Circadian Rhythm

In *E. gracilis* a pronounced circadian rhythm has been detected which governs many biochemical, physiological and behavioral parameters (Wolff and Künne 2000; Mittag 2001; Kiyota et al.

2006; Bolige and Goto 2007). Also the precision of gravitactic orientation follows a very distinct rhythm (Lebert et al. 1999a). When the cultures are kept in constant light the individual cells are in different phases of their rhythm so that no circadian changes in behavior are detected (Nasir et al. 2014). But when the cultures are exposed to a light/dark rhythm the gravitactic orientation follows a very distinct pattern with a minimum in the dark phase, an increase before the onset of light and a peak in the precision of orientation in the early afternoon (Häder and Lebert 2001b). This change in the expression of gravitaxis is not induced by the light or dark phase since the increase in precision of orientation occurs even before the light is switched on. It is rather an internal rhythm which is entrained by the light/dark cycle. This rhythm even persists when cells, which had been previously entrained, are transferred into constant light (Lebert et al. 1999a; Häder and Lebert 2001b). In parallel to the precision of gravitactic orientation, the form factor changes with cells being more elongated during daytime and more rounded at night. Also the swimming velocity increases in synchrony with the precision of gravitactic orientation. In addition, the concentration of intracellular cyclic adenosine monophosphate (cAMP) increases in parallel with the increase in gravitactic orientation (for the involvement of cAMP in the gravitactic signal transduction chain see below). In a follow-up publication the effect of shorter rhythms was investigated (1:1 h, 2:2 h, 4:4 h, 6:6 h and 8:16 h) (Richter et al. 2014). It was amazing that gravitactic orientation even followed the shortest rhythm. However, in this study graviorientation decreased after the light was switched on and increased after the beginning of the dark phase. The number of motile cells increased during the dark phase while the swimming speed was higher in the light phase. In contrast, during the light period the precision of gravitaxis increased while motility and velocity decreased after some hours of irradiation.

In *Chlamydomonas* the addition of a red background light strongly increases gravitaxis which is further augmented by the absence of calcium ions in the medium (Sineshchekov et al. 2000). In contrast, a blue light flash strongly decreased the precision of gravitactic orientation and could even reverse the direction of negative into positive gravitaxis. The spectral dependence of this response suggests the involvement of the photoreceptor for phototaxis, which is assumed to be a chlamy-rhodopsin (Kianianmomeni and Hallmann 2014).

12.6.2 Change in Gravitactic Orientation

Older cultures of *Euglena* (in stationary growth phase) show a pronounced negative gravitaxis and swim upward. In contrast, young cultures (shortly after inoculation, logarithmic growth phase) show a positive gravitaxis. The ecological reason for this differential behavior has not yet been revealed, but it could be speculated that in older cultures the concentration of nutrients is lower or the concentration of paramylon is different. However, it came as a surprise to find that the precise positive gravitaxis in young cultures was completely reversed by the application of heavy metals such as copper, mercury, cadmium or lead (Fig. 12.6) (Stallwitz and Häder 1994). Later on it could be shown that exposure to high UV and visible irradiances reverses the negative gravitaxis in older cells into a clear positive one (Ntefidou et al. 2002b; Richter et al. 2002c). The responsible wavelength band is in the UV

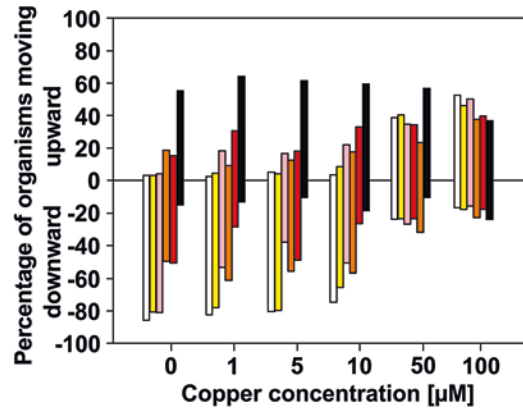

Fig. 12.6 Reversal of gravitactic orientation in young cells from downward (positive) to upward (negative) after the application of copper. The bars indicate (from *left* to *right*) a culture age of 4, 5, 6, 7, 8 and 11 days. Redrawn after (Stallwitz and Häder 1994)

and blue region of the spectrum. The sign change of gravitaxis is not brought about by an influence of the photoreceptor or the photosynthetic apparatus, because colorless and blind mutants (which do not possess a photoreceptor for phototaxis) as well as the colorless and non-phototactic *E. longa* also showed this reversal in movement direction. Rather it is mediated by reactive oxygen species (ROS) as shown by the fluorescence of the probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFA-DAL). Removing the oxygen by flushing the cells with nitrogen largely blocked this effect (Ntefidou et al. 2002a). Also the application of reduced dithionite suppressed the sign change (Richter et al. 2003b, 2003d). Likewise, the addition of Trolox, potassium cyanide or ascorbic acid, known scavengers of ROS, largely inhibited the sign reversal (Richter et al. 2003d). Cyanide blocks the cytochrome-c-oxidase which could hint to a possible role of hydrogen peroxide. In contrast, application of hydrogen peroxide induced a gravitactic sign change even in the absence of external stressors. It was concluded that the change in the sign of gravitaxis is induced by ROS, most likely hydrogen peroxide, which is known to be produced e.g. by the mitochondrial cytochrome-c-oxidase. Longer exposure to solar radiation reduced the precision of gravitactic orientation (Häder and Liu 1990b; Richter et al. 2002c).

The response of gravitaxis to stress factors such as heavy metals, but also herbicides, organic pollutants etc. is very sensitive (Ekelund and Aronsson 2004; Pettersson and Ekelund 2006; Streb et al. 2006; Azizullah 2011; Azizullah et al. 2011a, 2011b, 2011c). Therefore organisms such as *Euglena* are being used in bioassays based on image analysis of tracked cells. In addition to motility, swimming velocity and cellular form, the gravitactic orientation is used as an endpoint to monitor pollution and toxicity in drinking water, waste water and natural ecosystems (Ahmed 2010; Ahmed and Häder 2011; Azizullah et al. 2013).

E. gracilis is a freshwater organism, but tolerates moderate salinity. When exposed to increased salt concentrations (5–19 g/L) the percentage of motile organisms in a population, the swimming velocity and the precision of gravitactic orientation decreased (Richter et al. 2003a). At a concentration of 15 g/L those cells which were still motile switched from negative to positive gravitaxis. This positive gravitaxis persisted for several hours or days even when the cultures were returned to zero salinity. At very high salt concentrations (20 g/L) the cells entered the so-called palmella stage characterized by immobilized cells surrounded by a mucus layer (Karnkowska-Ishikawa et al. 2012). At this time the cells lost most of their carotenoids. When they recovered from this stage after being transferred into a salt-free medium the cells became motile again and positive gravitaxis persisted (Richter et al. 2003a).

Exposure of swimming *E. gracilis* to high-energy carbon irradiation or ^{60}Co gamma-rays (1–200 Gy) resulted in a decrease of the precision of negative gravitaxis (Sakashita et al. 2002c, 2002b). Motility was not affected, but the swimming velocity decreased during exposure to ionizing radiation. A calculation of the effective doses indicated that ionizing radiation in low Earth orbits would not affect gravitaxis in *Euglena*, but during solar flares there would be an inhibition of the orientation in low Earth orbits. It is interesting to note that the inhibition of negative gravitaxis by exposure to gamma-ray irradiation could be partially prevented by the application of 100 µM Trolox C as in the case of high salt stress (see above) indicating that the inhibition by gamma-rays may be brought about by the formation of free radicals (Sakashita et al. 2002a).

12.7 Gravireceptor in *Euglena* and Sensory Transduction Chain

Let us assume the hypothesis is correct that the intracellular content of the *Euglena* cell presses onto the lower membrane and activates a gravireceptor (Häder et al. 2005b); then the next logical question is: what is the gravireceptor? Many organisms–from bacteria to vertebrates–posses mechano-sensitive ion channels (Balleza and Gómez-Lagunas 2009; Jarman and Groves 2013). These pore proteins can be opened by minute

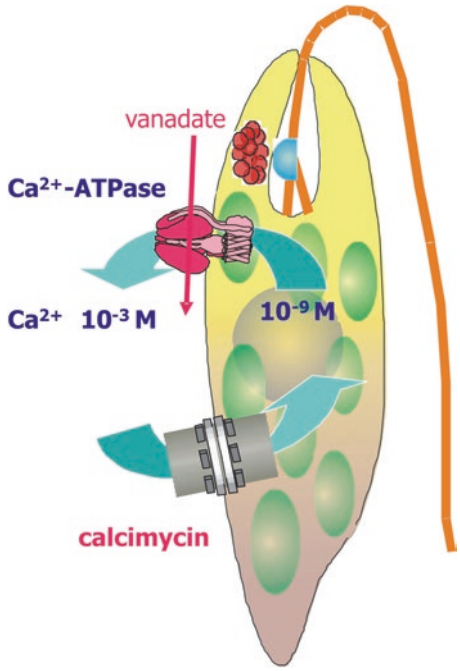

Fig. 12.7 Model of a *Euglena* cell showing the ATPase which pumps Ca^{2+} out of the cell. This pump can be blocked by vanadate. In the lower part the insertion of the ionophores A23187 is indicated which allows the passive leakage of Ca^{2+} from the outside

mechanical forces and allow the influx of ions. This passive influx follows an ion gradient across the membrane which had been previously established by active, energy-consuming ion pumps. *Euglena* uses a number of different ion pumps such as a calcium-specific ATPase which pumps Ca^{2+} out of the cell so that a resting internal concentration of about 10^{-9} M is established which is about six orders of magnitude lower than in the surrounding pond water (Weisenseel and Meyer 1997). This Ca^{2+} pump can be blocked by vanadate (Fig. 12.7). As a consequence the Ca^{2+} ion gradient across the membrane decreases and less Ca^{2+} flows in when the mechano-sensitive ion channels open upon gravistimulation and as a result the precision of gravitactic orientation decreases with increasing vanadate concentrations (Fig. 12.8a) (Lebert and Häder 1996; Lebert et al. 1997; Häder et al. 1999). Alternatively, an artificial ionophore (calcimycin, A23187) can be incorporated into the membrane which allows a passive leakage of Ca^{2+} ions into the cell which also reduces the Ca^{2+} gradient across the membrane. As a result the insertion of the ionophores decreases the precision of gravitactic orientation (Fig. 12.8b) (Lebert et al. 1996, 1997). Finally the mechano-sensitive channel itself, which allows the Ca^{2+} influx during gravistimulation, can be blocked by the application of gadolinium, which has a similar size as the Ca^{2+} ion and is thought to hinder the Ca^{2+} influx; also this measure impairs the gravitactic orientation (Häder and Hemmersbach 1997). Furthermore, addition of potassium or cadmium nitrate to the medium strongly impaired gravitaxis in *Euglena* (Fig. 12.8c) (Lebert et al. 1996, 1997). Also in *Chlamydomonas* mechano-sensitive channels have been found (Yoshimura 2011). A mutant which lacks these channels was found not to be capable of gravitaxis (Häder et al. 2006).

Due to the small size and low difference in the specific densities between the outer medium and the cellular content the force pressing onto the assumed mechano-sensitive ion channels is minute. Using a measured cell density of 1.05 g/mL the force can be calculated according to eq. (4) (Björkman 1992)

$$F = V \times g_n \times \Delta\rho \qquad (12.5)$$

where F = force [N], V = volume [L], g_n = acceleration [m s^{-2}] and $\Delta\rho$ = specific density difference between cell body and medium [kg/L]. Applying the values of *Euglena* to this equation the resulting force on the lower membrane is between 0.57 and 1.13 pN depending on the cell volume and the specific density (Lebert et al. 1999b).

If the hypothesis is true that the first step in the gravitactic signal transduction chain is a passive influx of Ca^{2+} along a previously established ion gradient by gating a mechano-sensitive ion channel it should be possible to visualize the increase in internal Ca^{2+} inside the cell. This can be done by loading a fluorescent chromophore such as Calcium Crimson into the cells which emits a fluorescence when binding to Ca^{2+} ions (McLachlan et al. 2012). In order to avoid the intensive chlorophyll fluorescence of green *Euglena*, *E. longa* was chosen, which also shows a pronounced negative gravitaxis. The fluorescence indicator was loaded

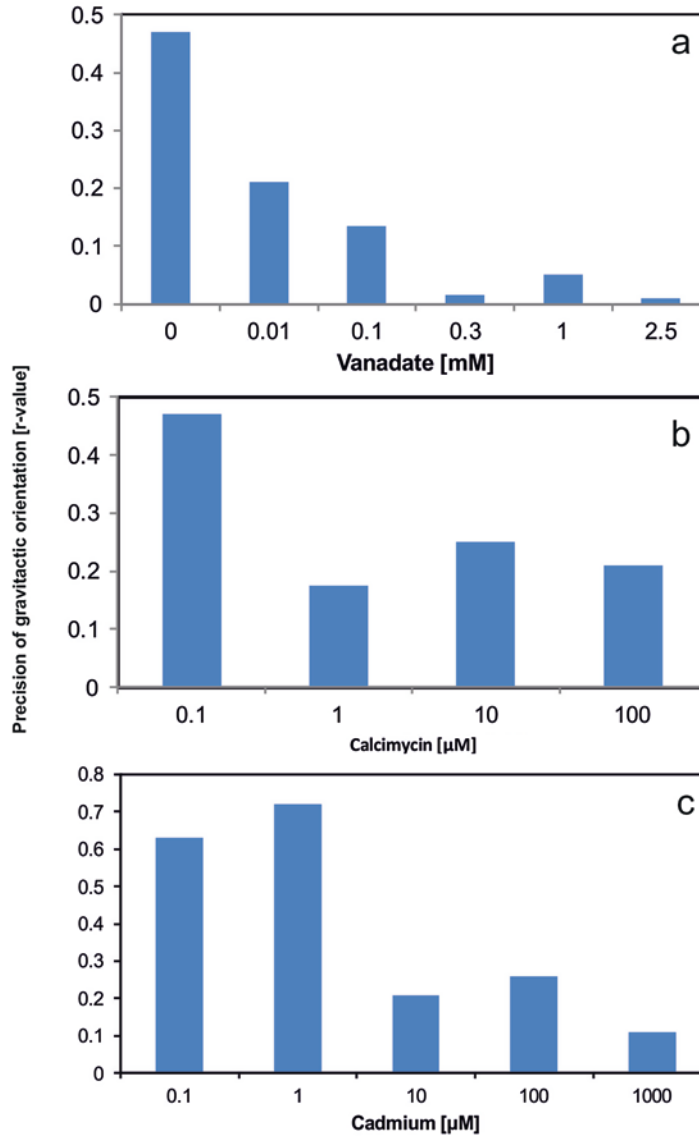

Fig. 12.8 (**a**) Precision of gravitactic orientation (r-value) in dependence of the vanadate concentration in the medium. (**b**) Effect of the ionophore calcimycin (A23187) on the precision of gravitactic orientation in *Euglena*. Redrawn after (Lebert et al. 1996). (**c**) Inhibition of gravitaxis by cadmium; redrawn after (Stallwitz and Häder 1994)

into the cells by electroporation, and the cells were allowed to orient themselves in a vertical cuvette. Then the cuvette was turned upside down so that the cells had to reorient; this was controlled by cell tracking. During reorientation a strong calcium signal was observed at the tip of the cell near the reservoir. This result was verified with the gravitactic competent, but colorless *Euglena gracilis* mutants 1F, 9F and FB (Häder and Lebert 2001b).

Using a spectrofluorometer, the fluorescence intensity could be quantified in *E. longa* cells loaded with Calcium Crimson after gravitactic stimulation (mixing the cells with a suction pipette after they had become precisely oriented (Richter et al. 2001b). The excitation beam was set to 580 nm and the fluorescence emission was monitored in the wavelength band 610–650 nm. During reorientation the fluorescence intensity rose with a short delay which corresponds with the delay found for the reorientation kinetics. Changes in Calcium Crimson fluorescence were also recorded during the microgravity and hypergravity phases produced by parabolic

airplane maneuvers (see below) (Richter et al. 2002d). In order to further prove the calcium-influx hypothesis we measured the Ca^{2+} fluorescence signal after gravitactic stimulation in the presence of gadolinium which blocks the inward Ca^{2+} current through the mechano-sensitive ion channels; indeed, the Calcium Crimson fluorescence was much lower than in the uninhibited control. Gadolinium has been found to inhibit stretch-activated ion channels (Sachs and Morris 1998). In contrast, addition of caffeine (10 mmol/L), which increases the cAMP concentration in *E. gracilis* (Porst 1998; Tahedl et al. 1998), reproducibly increases the calcium fluorescence in *E. longa* (Richter et al. 2003c) and in the 1F mutant of *E. gracilis* (Richter et al. 2001b). These results were confirmed on a parabolic flight using a sounding rocket (MAXUS 3). For this purpose a module was designed consisting of two identical epifluorescence microscopes with observation chambers filled with *E. longa* suspensions mounted on a centrifuge inside the payload (Häder and Lebert 1998). During launch the microscopes were rotated at 2 Hz to avoid cell sedimentation out of the field of view caused by the high acceleration forces during takeoff. During the parabolic flight the cells could be observed by an on-line video downlink. Focusing and lateral movement of the observation chamber could be realized by telecommands. During the flight three defined centrifugal acceleration steps (0.1, 0.2 and 0.3 g) were applied during the total time of 840 s in microgravity (Häder and Lebert 1998). After onset of microgravity the cells showed an intermediate Ca fluorescence signal. After acceleration by the centrifuge the calcium signal increased steeply and decreased again to the initial level about 10 s after the centrifuges had stopped.

In addition to missions on the American Shuttle, the ISS, satellites and rockets, parabolic flights on airplanes provide valuable opportunities to study the behavior of organisms under microgravity (Volkmann et al. 1991; Richter et al. 2002d; Krause et al. 2006; Bock et al. 2007; Strauch et al. 2010; Studer et al. 2011; Grosse et al. 2012). During the flight maneuver phases of 1 g are followed by an increase in acceleration to 1.8 g when the pilot pulls the airplane up. Afterwards the airplane enters a free-fall phase and the cells are exposed to microgravity for 20–25 s, followed again by a 1.8 g period. During the 1.8 g acceleration periods the Ca^{2+} fluorescence increases as compared to the 1 g phase, and during the microgravity phases it decreases.

The hypothetical mechano-sensitive calcium ion channels triggered by gravity cannot be located all around the cell but must be localized in a specific region in order to be fired when the cell is in the correct position for a course correction (Häder et al. 1997). When the cell is swimming upward during negative gravitaxis the channels should not be activated, thus, we would expect them to be located in the front end of the cell. Furthermore, during horizontal swimming these channels should be triggered when the flagellum points downward so that, when it swings out, it can initiate an upward turn of the front end. In that case the cell content should exert pressure on the channels. Thus, the location has to be at the front end underneath the trailing flagellum (Häder et al. 2005a). By repetitive reorientation maneuvers the cell steers into a vertical swimming direction.

The assumed Ca^{2+} influx into the cell during reorientation should result in a change in the electrical potential difference between inside and outside of the cell. Measuring the intracellular potential of *Euglena* cells using microelectrodes has failed up to now (Häder 2003). An alternative possibility to determine the membrane potential is measuring the electrochromic absorbance bandshift of natural components of the cell such as carotenoids (Armitage and Evans 1981). Another option is to load the cells with an artificial dye, such as Oxonol VI, which changes its distribution between the cytoplasm and the membrane accompanied with an absorption change between 590 and 610 nm when the membrane potential changes (Haugland 1997). We constructed a photometer with two sets of LEDs with maximal emission at 590 and 610 nm, respectively. After passing the beams through the *Euglena* suspension loaded with Oxonol VI, the absorption was measured alternatively at the two wavelengths by an array of four phototransistors as sensors and the ratio was calculated which

serves as a proxy for the membrane potential (Richter et al. 2001a). In parallel the gravitactic orientation was measured by cell tracking (Lebert and Häder 1999a). At the beginning of the experiment the cells were disturbed by gentle mixing and then allowed to reorient. During the cellular reorientation the membrane potential transiently increased (became more positive) which can be explained by the influx of Ca^{2+} ions through the membrane-sensitive ion channels. Sequestering calcium ions in the outer medium by the application of EGTA affected gravitactic orientation and abolished the membrane potential change measured with oxonol VI as shown during a parabolic flight campaign (Richter et al. 2006).

If indeed gravitactic orientation is coupled with a change in the membrane potential, it should be possible to interfere with the signal transduction mechanism by experimentally manipulating the membrane potential. This can be achieved by using a lipophilic cation such as triphenylmethyl phosphonium ($TPMP^+$) which penetrates the membrane and thus reduces the internal negative potential (Finichiu et al. 2013). Application of $TPMP^+$ at micromolar concentrations effectively reduces the precision of orientation in *E. gracilis* (Lebert et al. 1996).

12.8 Molecular Biology to Disentangle the Gravitactic Signal Transduction Chain

The existence of mechano-sensitive channels in *Euglena* was confirmed by molecular biological methods. In *Saccharomyces* a gene for a mechano-sensitive channel has been sequenced. When we employed several primers against conserved regions of this gene during PCR on the cDNA extracted from *Euglena*, four sequences of different lengths were detected corresponding to the homologue sequence in *Saccharomyces* (Häder et al. 2003). More than 1500 PCR products from many potential mechano-sensitive channel families were isolated, cloned in plasmids and sequenced (Häder et al. 2009). These proteins belong to the families of MID1 (*S. cerevisiae*), MEC (*C. elegans*), MSCL (*E. coli*), BNC1/BNAC (*H. sapiens*) and TRP (*D. melanogaster*, *H. sapiens* and others). However, most of these sequences coded for proteins were involved in other functions; but one sequence resembled the gene for a TRP channel. The TRP protein family is involved in many different physiological functions in the cell such as photoperception, nociception (pain sensation), thermosensation, tactile sensation, mechanosensing, taste, osmolar sensation and fluid flow sensation (Nilius and Owsianik 2011; Nilius et al. 2012). The assumed structure of the mechano-sensitive ion channel TRPC7 consists of six membrane spanning helices and the TRP pore (Häder et al. 2009).

One way of determining whether a specific gene product is involved in the signal transduction chain of gravitaxis is the application of RNA interference (RNAi) (Tuschl et al. 2014). In this process double-stranded RNA (dsRNA) induces a sequence-specific post-transcriptional gene silencing. Short RNA fragments (19–23 nucleotides) are introduced into the cells by electroporation. The injected RNA combines with the mRNA of the related protein in an antisense fashion and by this interferes with the function of the mRNA, effectively blocking translation and thus the synthesis of the corresponding protein. Using degenerated primers designed according to the pore region of the mechano-sensitive channel four PCR transcripts were isolated from *E. gracilis*. These products were cloned, sequenced and analyzed with BLAST. Three of the products did not resemble TRP channels while the fourth had a low similarity to the C-terminal end of TRP channels (Häder et al. 2009). Applying RNAi to the first three PCR products did not affect gravitactic orientation, but the forth effectively inhibited gravitaxis. This effect lasted for up to 30 days.

After characterization of the primary gravireceptor to be a transient receptor potential-like (TRP) channel protein, the next logical question was: What do the Ca^{2+} ions do inside the cell during gravitactic reorientation? Ca^{2+} ions are universal messengers in many organisms from bacteria to vertebrates and bind to proteins called calmodulins (Panina et al. 2012; Adler 2013) which usually consist of about 150 amino acids

and normally have four binding sites for calcium ions (Means 2013). Each calcium binding motif consists of a 12 amino acids loop flanked on both sides by a 12-residue α-helix which undergoes a conformational change upon calcium binding. Following the hypothesis that Ca^{2+} ions bind to a calmodulin during gravitactic orientation in *Euglena* we searched for a calmodulin gene in this organism. To our surprise we did not find one gene but at least five calmodulin genes (Daiker et al. 2010). The amino acid sequence coded by one of these genes (CaM.1) was already known from *Euglena* (Toda et al. 1992). We sequenced the genes of all five calmodulins (CaM.1–CaM.5) isolated from *E. gracilis*. All five calmodulin gene sequences differ from each other, but the EF motif, typical for calmodulins, is conserved in all five genes (Daiker et al. 2010).

Using the RNA inhibition method described above we blocked the protein synthesis of each calmodulin separately by electroporating the dsRNA of each calmodulin gene in separate *Euglena* populations by electroporation. RNAi against CaM.1 resulted in severe abnormalities of the cell form (Daiker et al. 2010). Even though the flagellum was visible the cells did not swim but showed only euglenoid (crawling) movement (Häder et al. 2009). Cell division was impaired and the cells showed abnormal outgrowths. By means of RT-PCR it could be confirmed that no mRNA of the blocked calmodulin was expressed (Daiker et al. 2010).

Application of RNAi against CaM.2 also resulted in euglenoid movement and cell abnormalities but only transiently for several days (Daiker et al. 2010). After about 1 week the cells recovered and swam normally, but they did not show any gravitactic orientation. RNAi against the remaining calmodulins (CaM.3–CaM.5) had no visible effect on the cell form, swimming behavior or gravitaxis. These surprising results indicate that only one of the identified calmodulins (CaM.2) is involved in the signal transduction chain of gravitaxis. In *Chlamydomonas* a calmodulin was detected in the flagella (Gitelman and Witman 1980). In *Euglena* the previously known calmodulin (CaM.1) was found underneath the pellicula (Toda et al. 1992) where it may be responsible for the cell form and the euglenoid movements which might explain the effects of RNAi of CaM.1 on motility and cell form abnormalities.

What is the next step in the gravitactic signal transduction chain? Masakatsu Watanabe and Mineo Iseki had shown that the light signal for photophobic responses is absorbed by a novel PAC photoreceptor molecule, a light-activated adenylyl cyclase, which produces cyclic AMP (cAMP) from ATP when activated by light (Iseki et al. 2002) (cf. chapter on photomovement in *Euglena*, this volume). Could it be possible that cAMP is also involved in the signal transduction chain of gravitaxis?

Preliminary experiments had shown that during the circadian rhythm in *E. gracilis* the intracellular concentration of cAMP increases in parallel with the precision of gravitactic orientation during the day (see above) (Lebert et al. 1999a). In order to prove the involvement of cAMP in the gravitactic signal transduction chain a very complex space experiment was devised for a flight on a sounding rocket (TEXUS 36) (Tahedl et al. 1998). Aliquots of 1 mL cell suspension were filled into 2-mL syringes which were connected by a tube with a second 2-mL syringe containing 1 mL ethanol, but separated by a rubber ball. A total of 112 of these assemblies were housed in frames and the fixative could be injected explosively into the cell suspension by hydraulic pistons at predetermined times during the sounding rocket flight. The frames were oriented radially on a centrifuge to provide three different radii to allow different accelerations at the same centrifugal speed. In addition to the controls, some of the experiments contained 1 mM gadolinium chloride which is an inhibitor of mechano-sensitive channels (Yang and Sachs 1989; Lebert and Häder 1996) or 10 mM caffeine, an inhibitor of phosphodiesterases (Lebert et al. 1997; Spoto et al. 1997; Häder and Lebert 2002). During the flight, individual groups of experiments (8–12 syringes in parallel) were activated so that the cells were fixed at different times and accelerations. The cAMP concentration inside the cells after a given exposure time to a given acceleration was quantified using a

radioimmunoassay and a scintillation counter. During microgravity the cAMP content of the cells was low and did not increase significantly at an acceleration of 0.08 g, which previous experiments had shown to be below the threshold for gravitaxis in *Euglena*. However, when accelerations of 0.12 g or 0.16 g were applied the cellular cAMP concentrations increased considerably (Tahedl et al. 1997). Gadolinium-treated cells showed a significantly lower increase in cAMP upon gravitactic stimulation (Tahedl et al. 1998). In caffeine-treated cells no increase in cAMP could be observed during stimulation, but caffeine-treated cells had about three times higher concentrations of cAMP in microgravity. Probably no further increase could be produced by the adenylyl cyclase. It is interesting to note that also in a motile slime mold an activated adenylyl cyclase has been found to be responsible for the control of movement and development (Renart et al. 1981; Sultana et al. 2012). Also the ciliate *Paramecium* has been found to involve cAMP in its gravitactic sensory transduction chain (Bräucker et al. 2001; Hemmersbach and Braun 2006).

In order to confirm the hypothesis of involvement of calmodulin and an adenylyl cyclase in the gravitactic sensory transduction chain a number of inhibitor studies were carried out. Trifluoperazine and fluphenazin as well as W7 are specific calmodulin inhibitors (Naccache 1985; Russo et al. 2014; Son et al. 2014) and were found to inhibit gravitaxis in *E. gracilis* (Häder et al. 2006). Likewise, indomethacine impairs the adenylyl cyclase (Wang et al. 2012) and decreases the precision of gravitactic orientation in *E. gracilis* (Richter et al. 2002b; Häder et al. 2006), while forskolin activates the adenylyl cyclase (Schwer et al. 2013) and augments gravitaxis in *Euglena* (Häder et al. 2006). Phosphodiesterase quenches the cAMP signal (Cai et al. 2015). When this enzyme is inhibited by caffeine, theophylline or IBM-X the cellular cAMP concentration increases (Cameron and Baillie 2012; Steck et al. 2014) which explains why e.g. application of caffeine or IBM-X enhances the gravitactic orientation (Streb et al. 2002). 8-Bromo-cAMP is an analog of cAMP which is not degraded by the phosphodiesterase but seems to have a similar function. Addition of this drug consequently augmented the precision of gravitactic orientation in *Euglena* (Lebert et al. 1997).

The final piece in the puzzle of the sensory transduction chain is how cAMP controls the flagellar activity which finally results in a course correction of the cell. cAMP has been found to activate a protein kinase A (Favaro et al. 2012) which could be responsible for the change in flagellar activity. Staurosporine is an effective inhibitor of protein kinases (Chang and Kaufman 2000). When applied to swimming *Euglena* the cells lost their ability of gravitactic orientation with increasing exposure time (Häder et al. 2010). 225 min after application a positive gravitaxis was observed (Daiker et al. 2011). This result reminds us of the fact that negative gravitaxis can be reversed into a positive one by several stress factors (see above). But the full meaning of this finding is not yet fully understood. Also, negative phototaxis was blocked by staurosporine with increasing exposure time (see chapter on photomovement in *Euglena*, this volume). This indicates that gravitaxis and phototaxis share the same last step in their sensory transduction chain. While the initial parts are different, in the end the production of cAMP activates a phosphokinase A which controls the flagellar reorientation.

Euglena possesses at least five isoforms of the protein kinase A (PK.1–PK.5) as has been found by using degenerate primers. RACE-PCR was used to reveal the full range of the RNA sequences and sequence the genes. Since these five genes differ in certain sequences it was possible to produce specific ds-RNA against the individual genes. These were inserted into different *Euglena* populations by electroporation for RNA interference. The resulting populations were tested for gravitaxis. RNAi of PK.1–PK.3 and PK.5 did not affect gravitactic orientation, indicating that these protein kinases were not involved in the gravitactic signal transduction chain (Daiker et al. 2011). In contrast, application of RNAi on PK.4 strongly affected the precision of gravitaxis up to several weeks. After about 22 days a positive gravitaxis was observed which reminds us of the fact that also staurosporine

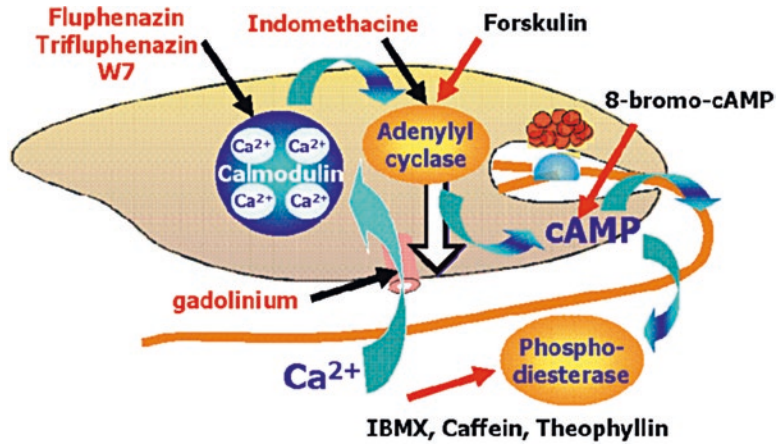

Fig. 12.9 The model shows the proposed signal transduction chain for gravitactic stimuli with the components involved as well as the inhibitors and enhancers

inverted the negative into a positive gravitaxis after prolonged application (see above). Furthermore, RNAi on PK.4 impaired phototaxis (see chapter on photomovement in *Euglena*, this volume). This again confirms that gravitaxis and phototaxis share the same last step—the activation of a protein kinase A by cAMP—in their sensory transduction chain.

Combining all these results we can construct a signal transduction chain for gravitactic orientation in *Euglena* (Fig. 12.9) (Häder 2010). The cell interior has a higher specific density than the surrounding medium. During horizontal swimming it exerts a pressure onto the lower membrane with the mechano-sensitive TRP channel whenever the flagellum points downwards during its helical path. The gating of the channel can be blocked by gadolinium. The inflowing Ca^{2+} binds to a specific calmodulin (CaM.2) which can be blocked by fluphenazin, trifluphenazin or W7. The activated calmodulin in turn activates an adenylyl cyclase. This can be inhibited by applying indomethacine, while forskolin augments its action. The adenylyl cyclase uses ATP to produce cAMP which in turn is broken down by a phosphodiesterase. 8-bromo-cAMP can substitute the cAMP function but is not broken down by the phosphodiesterase. IBMX, caffeine or theophylline inhibit the phosphodiesterase, so that the intracellular cAMP concentration is enhanced. All inhibitory or stimulatory actions are mirrored in impaired or augmented gravitactic orientation of *Euglena*.

Figure 12.10 shows the key players in the sensory transduction chain for gravitaxis and phototaxis which share the final element (protein kinase A) which is believed to control the reorientation of the flagellum. Whether or not calmodulin itself could activate the protein kinase A has not yet been revealed. The sensory transduction chain of the photoorientation starts with the activation of the PAC photoreceptor by light which results in the production of cAMP by the light-activated adenylyl cyclase which in turn is thought to activate the same protein kinase A, involved in graviperception. The components which have been blocked by RNAi to prove their involvement in the respective transduction pathways are indicated.

12.9 Conclusions and Outlook

Euglena gracilis uses a physiological gravireceptor for an active orientation with respect to the gravity vector of the Earth. The basic elements of the sensory transduction chain such as the mechano-sensitive transient receptor potential Ca^{2+}-conducting ion channel, the specific calmodulin (CaM.2) (Häder et al. 2009), the adenylyl cyclase and protein kinase (PK.4) (Daiker et al. 2011) have been identified. The number and localization of the TRP channels still need to be confirmed e.g. by immunofluorescence using polyclonal antibodies. Likewise, the location of the specific CaM.2, thought to be located inside

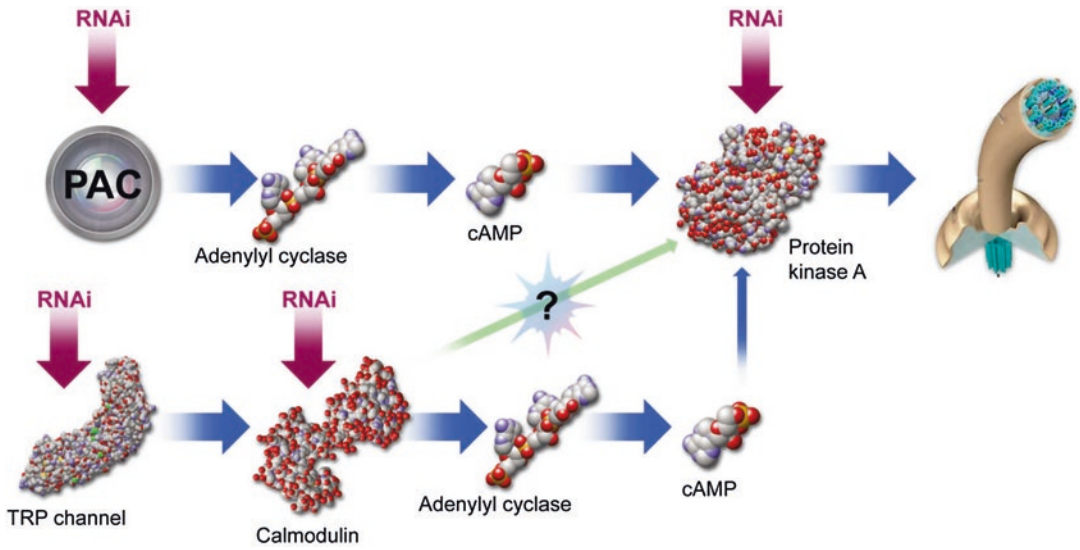

Fig. 12.10 Assumed sensory transduction chain of gravitaxis in the flagellate *E. gracilis*. When the pressure of the cell content activates a mechano-sensitive channel during horizontal swimming, calcium ions enter the cell passively along a previously established Ca^{2+} gradient and activate a specific calmodulin (CaM.2) which in turn induces an adenylyl cyclase to produce cAMP from ATP. This messenger activates a protein kinase A (PK.4) which is thought to provoke the change in the flagellar bending resulting in a reorientation response of the cell. The phototactic sensory transduction chain is included in this model showing the PAC photoreceptor which is a light-activated adenylyl cyclase. The produced cAMP is thought to activate the same protein kinase which is involved in gravitaxis. The molecular structures resemble typical members of the respective protein families. The structures of the specific proteins in *Euglena* are still unknown. Redrawn after (Häder 2010)

the flagellar membrane needs to be confirmed. Also the cellular positions of the adenylyl cyclase and phosphokinase (PK.4) need to be identified. Another open question is the nature of the adenylyl cyclase which is probably not identical with the adenylyl cyclase involved in phototaxis since this is part of the photoreceptor located in the paraxonemal body (Iseki et al. 2002). The protein kinase is hypothesized to phosphorylate proteins inside the trailing flagella. These proteins need to be identified to understand the molecular mechanism of flagellar reorientation. The potential involvement of the second flagellum, which does not extent out of the reservoir, as well as the axonemal rod (Bouck 2012), a specific element found in Euglenoid cells, need to be revealed.

Another open question concerns the control of the direction of gravitaxis. The existing results indicate that ROS are involved in changing the direction of gravitaxis (Richter et al. 2003d). However, how ROS operates on the molecular signal transduction chain to reverse the direction of movement still remains to be resolved.

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