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The Electrical Network of Maize Root Apex is Gravity Dependent

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Investigations carried out on maize roots under microgravity and hypergravity revealed that gravity conditions have strong effects on the network of plant electrical activity. Both the duration of action potentials (APs) and their propagation velocities were significantly affected by gravity. Similarly to what was reported for animals, increased gravity forces speed-up APs and enhance synchronized electrical events also in plants. The root apex transition zone emerges as the most active, as well as the most sensitive, root region in this respect.

Life on Earth has evolved under omnipresent and stable gravity forces which act on all living organisms of the planet. Such a permanent physical stimulus, influencing both growth and behaviour, has led to the evolution of gravity perceiving systems in almost any organism. This is true also in plants, where gravitropism plays a central role in the whole plant life cycle (see for example refs. 1–3).

Fast gravity perceiving systems, when the perception of a changing acceleration is rapidly transduced into electrical signals, have been documented from unicellular organisms[4] up to neuronal tissues[5,6]. In plants, numerous studies indicate that cell membranes, membrane proteins and membrane potentials are involved in the perception of gravity[7–9]. Typically, organismal responses to this environmental stimulus involve the modulation of the cell bioelectrical properties[10,11]. Numerous genes are up- and down-regulated in roots of plants exposed to microgravity[12–14]. In addition, roots exposed to microgravity change some of their behavioural features such as their responses to electric fields and light[15,16]. However, inherent root behaviour patterns, such as circumnutations and waving/skewing when grown on the agar plates, have turned out to be gravity-independent[17–18].

In animals, the properties of action potentials (APs) have been also found to be gravity-dependent. Experiments with isolated nerve fibres[19] and muscles[20] showed gravity-sensitive APs. For example, their propagation velocities and the frequency of their generation increased under hypergravity and decreased under microgravity conditions compared to the ground control. Alterations in ion channel permeability due to changes in gravity may be involved in these responses[21,22], indicating that gravity detection might be an intrinsic property of biological membranes and/or of their ion channels.

In higher plants, measurements of electrical potentials under gravistimulation (applying a rotation of 90° to the plant body) have been accomplished since the last century[23]. There is substantial evidence that the reorientation of plants in the gravity field induces a transient electrical activity (the so-called "geoelectric effect") (for a review see ref. 24). More recently, a number of investigations on the effect of gravity change on single cells, shoot and roots of plants have been performed. For example, fast changes (up to 17 mV) in surface potentials following gravitropic stimulation have been observed in soybean hypocotyls[25], while a transient of rapid surface potential of about 10 mV has been measured in bean epicotyls about 30–120 s after gravistimulation[26]. Concerning the root system, membrane hyperpolarization has been detected in the columella cells[7] and in the transition zone of the root apex[27] under gravistimulation. At the intracellular level, the correlation of different types of ion fluxes to gravistimulation has been also investigated. The plasma membrane (PM) and the endoplasmic reticulum (ER) seem to be involved in the sensing and transduction of gravity in plants[1,8]. Moreover, voltage-gated, mechano-sensitive, and ligand-activated ion channels (particularly Ca^{2+} channels), as well as proton pumps (e.g. the electrogenic H^{+}-ATPase), are involved in generation and maintenance of these bioelectrical potentials[24].

In all these investigations, the gravistimulation consisted of a changed orientation of the plants; namely, changes in the direction of the gravity vector. Are there any similar responses when analysing the effect of changes

Table 1 | Spike rate and spike duration recorded in the root apex in different gravity conditions. Data are means ± SEM; N = 6, 14, 12, 12 respectively for 0 *g*, 1 *g*, 2 *g* and 5 *g*; $p = 0.0042$ for spike rate and $p < 0.0001$, $F = 18.42$, $R^2 = 0.22$ for spike duration; different letters in superscript following values indicate statistical significance

Gravity condition	Rate, spikes per second	Duration, ms
0 *g*	0.23 ± 0.02^{b}	29.10 ± 1.66^{c}
1 *g*	0.35 ± 0.04^{c}	25.32 ± 0.54^{b}
2 *g*	0.30 ± 0.03^{bc}	20.22 ± 0.80^{a}
5 *g*	0.17 ± 0.02^{a}	19.90 ± 0.78^{a}

in the magnitude of gravity? Fast chemical responses to microgravity stress, involving oxygen and nitric oxide fluxes, have been reported in plants exposed to microgravity during parabolic flight experiments[28]. In a previous study, we used for the first time a multi electrode array (MEA) system to monitor the electrical activity of root cells during parabolic flight observing fast electrical signals whose frequency of firing rate influenced by the gravity condition[29]. In the present paper, we have compared the effects of microgravity and hypergravity on plant roots electrical activities recorded during parabolic flights (0 g) and on a large diameter centrifuge (2 g and 5 g). The aim of this study was to achieve a better understanding on how and why the AP properties are gravity-dependent in plants and to compare these results to those available in literature for animal tissues.

Results

Preliminary analysis showed no differences between neither the flight control and ground control (1 *g*), nor between 2 *g* increased gravity level obtained in the large diameter centrifuge (LDC) and that achieved (1.8 *g*) during parabolic flights (Fig. S1). Nevertheless, for the second case, we decided to show and discuss only results obtained in the LDC experiments because, due to the larger number of replicates and the longer experimental sessions, we could reach a stronger statistical analysis.

The data analysis revealed interesting differences in the AP spike rate occurring in altered gravity conditions (i.e. 0, 2 and 5 *g*) when compared with controls (i.e. 1 *g*) (Table 1). In particular, the electrical activity recorded in 0 *g* and 5 *g* was greatly reduced when compared with 1 *g* ($p = 0.0042$). On the contrary, the effect of 2 *g* on spike rate was smaller and not statistically significant. This is perhaps due to the fact that plants normally undergo conditions of slightly increased forces, in particular with respect to their root system (for example see the temporary increase of pressure exerted on the surface of the ground by the passage of animals or machines), while, on the other hand, 0 *g* is a condition that they have never experienced throughout their evolution. In accordance with what we observed in our previous study[30], the TZ exhibits a higher rate of APs than the MZ, for all the gravity conditions (Fig. 1). Differences between the spike rate of the TZ and MZ were statistically significant in all gravity conditions tested (one-tailed $p \leq 0.05$), with the biggest gap existing in the case of 0 *g* and 1 *g*, and declining in the two hyper-*g* conditions.

In order to better characterize the nature of the electrical activity in the two regions of the root apex under different gravity conditions, we obtained cross-correlograms that showed the conditional probability of an AP at time $t_0 \pm t$ ($t = 50$ ms) on the condition that there is a reference event at time t_0, calculating the probability between APs that occurred at any pair of two electrodes covered by the root sample. The lag time t equal to 50 ms was chosen to be large enough to allow synchronized spikings involving the whole recording area (the maximum distance between two electrodes is 5 mm). In Fig. 2, cross-correlations of any possible pair of electrodes firing in synchrony is shown for the selected lag time t with a temporal bin of 10 ms. The analysis showed that under 0 *g*, the probability of correlated events is lower than in control experiments. In fact, only a few simultaneous events were observed. On the other hand, under increased gravity, and in particular in the case of 5 *g*, the occurrence of correlated events was higher. The highest peak at $t = 0$ observed in these cases shows that increased gravity conditions enhance synchronized electrical activity. Furthermore, we performed the same analysis for the study of synchronized events comparing pairs of electrodes belonging to two different groups, one in contact with the TZ, the other one with the MZ of the root apex. Such analysis showed similar probability of synchronizations between sites of the TZ in all gravity conditions, while cross-correlations between sites of the TZ *versus* MZ, and between paired sites of the MZ increased with increasing *g* level (Fig. 3). Performing this analysis, we found the highest peak at $t = 0$, showing that increased gravity conditions enhance synchronized electrical activity between cells of the MZ as well as a certain probability of synchronized spiking involving both regions within the root apex.

Higher-temporal-resolution analysis of each frame composing synchronized events allowed for the visualization of an impulse as it spread across the root apex. For the analysis, all signals spreading from the TZ to the MZ and *vice versa* were considered. The velocity of signal propagation in 0 *g* was lower than in control conditions (Fig. 4), especially at the beginning of the spread ($p = 0.0003$). On the contrary, 2 *g* and 5 *g* conditions had the opposite effect on the speed of propagation of signals in that they travelled significantly faster from the beginning of the spread (about 377 mm/sec and 510 mm/sec respectively for 2 *g* and 5 *g* at 0.5 mm from the origin of the signal, in respect to about 247 mm/sec and 110 mm/sec for 1 *g* and 0 *g*) to sites more distant from the origin of the signal. In fact, at a

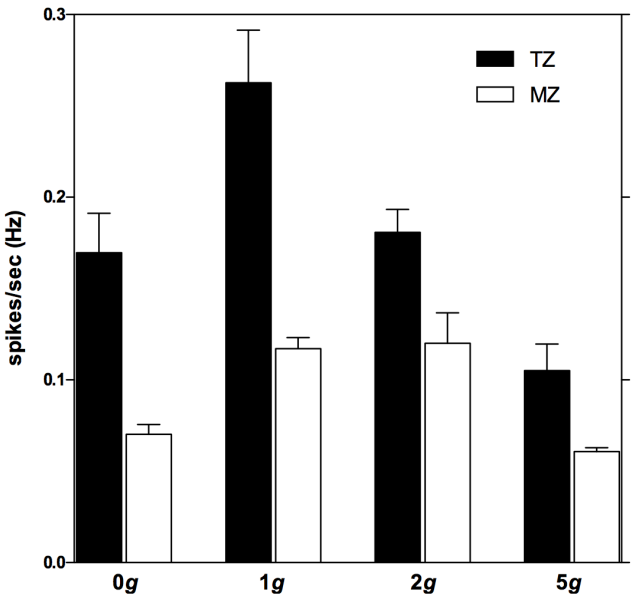

Figure 1 | Spike rate recorded in two regions of the root apex, the transition zone (TZ) and the mature zone (MZ). Differences are significant with one-tailed $p_{(0\ g)} = 0.0010$, $p_{(1\ g)} = 0.0006$, $p_{(2\ g)} = 0.0500$, $p_{(5\ g)} = 0.0196$ for, respectively, 0 *g*, 1 *g*, 2 *g* and 5 *g*. Data are means ± SEM; N = 6, 14, 12, 12 respectively for 0 *g*, 1 *g*, 2 *g* and 5 *g*.

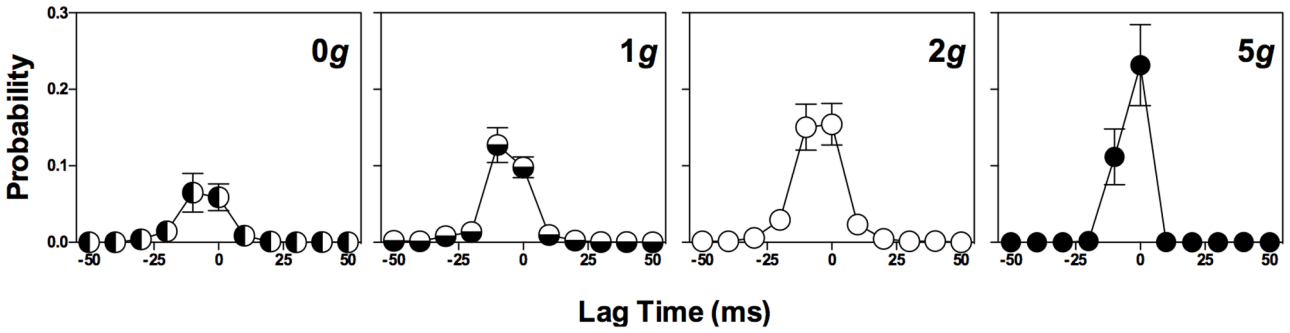

Figure 2 | Cross-correlograms of synchronized events. The probabilities that an AP occurring a t_0 is preceded or followed by other signals within the lag time $t = 50$ ms for different gravity conditions (bin = 10 ms) are shown. The highest the peak of the value of probability at $t = 0$, the more probable is the occurrence of synchronized electrical activity. Data are means ± SEM; N = 6, 14, 12, 12 respectively for 0 *g*, 1 *g*, 2 *g* and 5 *g*.

distance of 2.5 mm we measured signals propagating with an average speed two (in the case of 2 *g*) and five (in the case of 5 *g*) times greater than in 1 *g* and 0 *g*. Furthermore, under all gravity conditions, the velocity of the signal propagation decreased starting from the origin of excitation. This phenomenon is always observed in control experiments and it is supposed to be an intrinsic property of signal propagation in plant cells (for further reading, see ref. 30). In this study, we found out that such a decrease was much more pronounced in the 0 *g* condition, while in roots undergoing 2 *g* and 5 *g* accelerations, the signals appeared to propagate not only with a greater velocity but also maintaining such velocity for longer distances and, finally, being able to cover larger regions (up to 2.5 mm).

The duration of APs was calculated for all the spikes generated during the experiments and compared with those registered in control experiments. Table 1 shows that average durations recorded in roots monitored under 2 *g* and 5 *g* conditions were shorter with respect to control conditions (averaged AP duration were around 20 ms) while during 0 *g* APs duration was longer than in 1 *g* (around 30 ms *versus* around 25 ms).

Discussion

Our present results complement the previous one obtained using animal neuronal tissues and reveal that plant APs are gravity-sensitive in the same manner as already reported for animal APs[5,6]. We can conclude that APs in root tissues of higher plants and neuronal tissues of animals are gravity-dependent. This discovery urges several pressing questions. First of all, why are APs gravity-dependent? What are the consequences of this phenomenon? The gravity-sensitivity of APs is a very important issue not only for our future Space explorations but also for our understanding of the biological basis of APs in general. It fits well with several reports on psychological, cognitive and behavioral impairments in animals and humans under prolonged microgravity[31–34]. Moreover, there are still several unknowns associated with the APs[35], and the general gravity-sensitivity of APs might provide some important clues in this respect too.

APs are initiated via changed activities of ion channels embedded within the plasma membrane and it is not surprising that these ionic activities are related to environmental responses both in animals and plants. In animals, Steinhardt and Alderton noted very close similarities between synaptic activities linked to the generation of APs and the plasma membrane repair processes based on endocytic recycling vesicles[36]. This would suggest that the evolutionary origins of APs might be traced back to ancient eukaryotic cells repairing damaged plasma membranes in response to environmental stress. In fact, Andrew Goldsworthy proposed already in 1983 that plant APs evolved in response to damaged plasma membranes[37]. Repair of the damaged plasma membrane is preceded and allowed by a large plasma membrane depolarization, and plant APs might originally serve to restore optimal membrane potentials. Later, during the evolution towards large multicellular organisms, plant APs were co-opted for rapid cell-cell communication in order to coordinate their cells[38–40]. A similar scenario can be envisioned for other multicellular organisms. Interestingly in this respect, synaptotagmins act as calcium sensors for synchronized exocytosis, a process which is involved both in plasma membrane repair[41,42] as well as in neurotransmission[43,44].

In plants, electrical currents across and along plant tissues are closely linked to ion transport and these electric fields also influence plant cell polarity[45]. Importantly in this respect, the highest electric activity is scored in the root apex transition zone[30], which also shows the most active ion transport as reported by a long series of studies on the transport of ions like potassium, hydrogen, calcium, etc.[46–48].

Interestingly, Ishikawa and Evans recorded very fast electric responses in transition zone cortex cells of mung bean root apices[27]. In maize root apices, cells of the TZ show not only the highest electric activities[30] but are also the most active in the polar transport of auxin[49] and require the greatest amounts of oxygen[50]. Importantly, this transition zone peak in the oxygen influx is sensitive to gravity[28,51] and, similar to the polar auxin transport, the oxygen influx peak is dependent on brefeldin A-sensitive endocytic vesicle recycling[47,52]. All this suggests that brefeldin A-sensitive endocytic vesicle recycling might represent a gravity-sensitive process which integrates gravity sensing with gravity responses (see also ref. 52 and ref. 53 for more discussion on this issue). Therefore, our present data support the scenario in which gravity-sensitive vesicle recycling underlies AP activities in plants (see also ref. 53).

Our results and concepts from root cells indicate that endocytic vesicle recycling is tightly coupled to mechanical stresses which the plasma membrane experiences at the upper and lower portions of root cells[52,53]. Protoplastic load, driven by the force of gravity acting on the protoplasmic weight, is too high at the physical bottom. This mechanical stress on the plasma membrane is relieved by adding more membrane material as the balance between exocytosis and endocytosis is shifted towards exocytosis. Increasing the gravity forces above 1 *g* pushes this imbalance further, requiring more activity to restore the mechanical balance of the plasma membrane. This makes synapses more active and plasma membrane more excitable. The opposite scenario explains why microgravity inhibits APs as well as electric activities.

It is very interesting to note that gravity amplifies and microgravity decreases circumnutations in *Arabidopsis thaliana* stems[17], and that Dionea leaf trap closure, which is dependent on APs, is found to be slower under microgravity and faster under hypergravity[54]. Besides, fast changes in the cytoplastic calcium concentration ($[Ca^{2+}]_c$) of plant cells have been associated to different gravity conditions, and a consistent gravity dependency of plant responses with gravitational acceleration has been shown[55,56]. This resembles the effects of gravity and microgravity on plant APs reported here. Concerning the changes in circumnutations, it is worth noting that glutamate

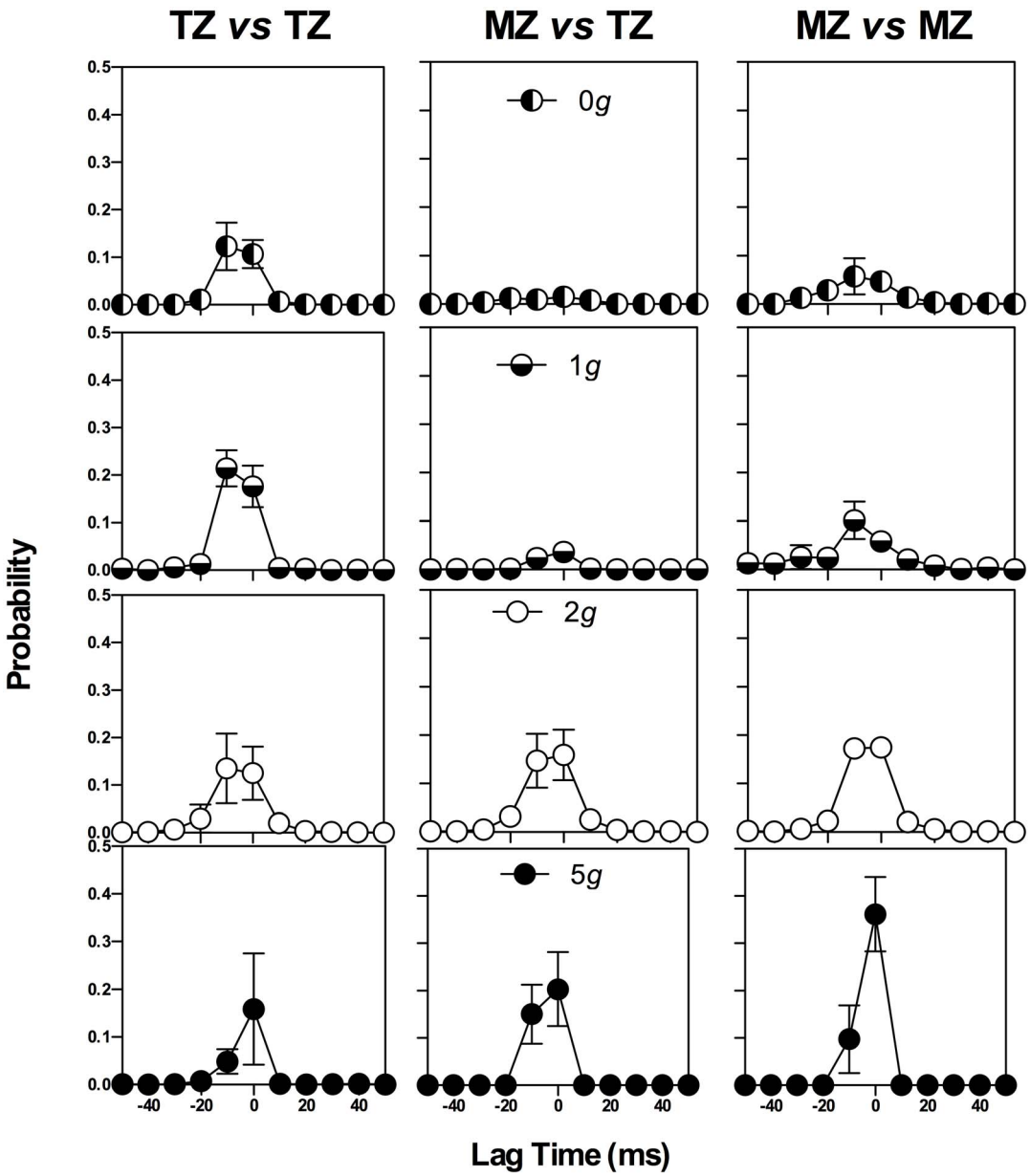

Figure 3 | Cross-correlograms of synchronized events. The probabilities that a spike (AP) occurring a t_0 is preceded or followed by other signals within the lag time $t = 50$ ms for different gravity conditions (bin = 10 ms) are shown. Pair wise of sites (electrodes) involved by the TZ, MZ or both zones of the root apex are considered. The higher the peak of the value of probability at $t = 0$, the more probable is the occurrence of synchronized electrical activity. The electrical activity of cells of the TZ has similar probability of synchronized events in all gravity conditions, while different gravity levels affect the cross-correlations between couples of sites of the TZ *versus* MZ, and of the MZ, where they show to increase with increasing *g* level. Data are means ± SEM; N = 6, 14, 12, 12 respectively for 0 *g*, 1 *g*, 2 *g* and 5 *g*.

induces APs not only in animals but also in plants[30,57]; and these glutamate-induced APs control circumnutations of *Helianthus annuus* stems[57,58]. In Arabidopsis, 20 glutamate receptors are expressed and their neuronal mode of action is well-conserved between animals/humans and plants[52,59,60]. It emerges that APs control not only circumnutations but perhaps other plant organ movements. This attractive scenario will be tested in our future studies. As plants evolved movements of their organs and their plant-specific APs convergently with animals, the gravity-dependency of APs both in animals and plants suggests some fundamental nature of this phenomenon.

Methods

Plant material and MEA set up. Caryopses of *Zea mays* L. cv. Gritz were soaked overnight in aerated tap water and placed between damp paper towels in Petri dishes. Dishes were incubated vertically at 26°C for 48 h, and seedlings used after roots reached a length of around 3 cm.

Electrical recording was carried out following the method detailed in Masi et al. (2009)[30]. In brief, longitudinal sections from the primary root tip were cut at 350 μm thickness and were stored submerged for 1–2 hours in 5 mM $CaCl_2$ (pH 6.5) at room temperature (22°C) before recording. The assessment of root cell injury with propidium iodide was not performed since previous studies[30] have shown negligible damages due to the cut and/or to the persistence of the tissue slice on the MEA surface during the recordings. Besides, such damage was expected to be present in equal intensity in all the samples. For recording, slices were gently transferred to a 6 × 10 multielectrode array with interelectrode distance of 500 μm and electrode diameter of 30 μm (MCS, Multi Channel Systems, Reutlingen, Germany). The electrodes (59 for recording the electrical activity plus 1 used as internal reference) are coated with porous titanium nitride to minimize the impedance and to allow for the recording of APs at a high signal to noise ratio (Fig. 5*A*). The array covered about 80% of the root apex, including the meristematic and transition zones, as well as part of the mature zone. Photographs taken under a microscope, before and after recording sessions, confirmed the localization of the recording electrodes. Experiments were performed

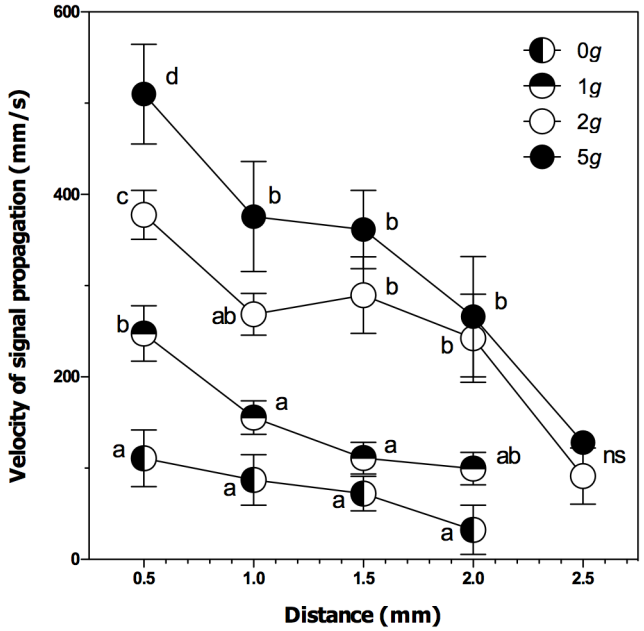

Figure 4 | Velocity of spreading electrical events. Measurements are performed up to 2.5 mm from the origin of the signal in root apices in different gravity conditions (all synchronized events collected for each sample have been used). Data are means ± SEM; N = 11, 49, 51, 88 respectively for 0 *g*, 1 *g*, 2 *g* and 5 *g*; $p_{(0.5\ mm)} = 0.0003$, $F_{(0.5\ mm)} = 15.63$, $R^2_{(0.5\ mm)} = 0.81$; $p_{(1.0\ mm)} = 0.0038$, $F_{(1.0\ mm)} = 8.22$, $R^2_{(1.0\ mm)} = 0.69$; $p_{(1.5\ mm)} = 0.0002$, $F_{(1.5\ mm)} = 16.15$, $R^2_{(1.5\ mm)} = 0.82$; $p_{(2.0\ mm)} = 0.0048$, $F_{(2.0\ mm)} = 9.73$, $R^2_{(2.0\ mm)} = 0.79$; different letters indicate statistical significance.

in a bath solution containing 5 mM $CaCl_2$ (pH 6.5) and the temperature was set at 26 ± 1°C with a temperature controller (TC01, MCS). The slices were taped onto the MEA using an adhesive transpiring and water resistant tape (3M™ Micropore Surgical Tape). This tape mediates a good adhesion of the tissue to the MEA surface, while at the same time allowing for the superfusion of the tissue. The chamber was completely filled up with the measuring solution and sealed using a special lid (Fig. 5*B*). This setup prevented any solution spillage and assured a stable contact between the sample and the electrodes in ground and altered gravity conditions.

Parabolic flights experiment design. The experiments were conducted during the 46th ESA parabolic flight campaign performed at Novespace in Bordeaux (France). On each parabola, there is a period of increased gravity (1.8 *g*, 1 g = 9.81 ms^{-2}), which lasts for about 20 seconds immediately prior to and following a period of around 20 second of microgravity (<0.05 *g*). A flight campaign consists of three individual flights; around 30 parabolas are flown each flight, for a total of 90 parabolas. During each flight, two different root samples, assembled on ground, were carried on board and used to record the electrical activity; the sample was replaced after the first 15 parabolas. An accelerometer was used to record the evolution of

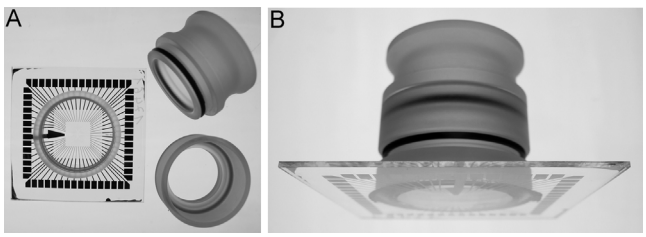

Figure 5 | The MEA chip used to record the electrical activity of maize root apex in gravity changing conditions. Sixty electrodes (59 + 1) are printed at the bottom (electrode diameter: 30 μm; inter-electrode distance: 500 μm). Each electrode detected signals from multiple cells. (*A*) Overview of a multielectrode array (MEA) and of the special lid used to avoid any spill of solution and assure a stable contact between the sample and the electrodes also in altered gravity condition. (*B*) The chamber fixed on the MEA filled with $CaCl_2$ 5 mM (pH 6.5) solution.

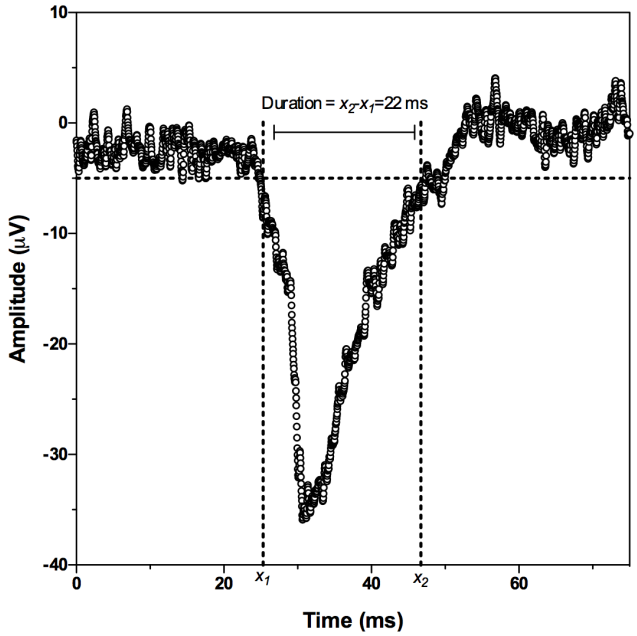

Figure 6 | Representation of one spike (AP). The procedure used for the measurement of the spike duration is shown.

gravity during the flight. For the statistical analysis, data of each day and experiment were grouped according to the class of acceleration.

Hypergravity experiment design with a LDC. Experiments were performed in the Large Diameter Centrifuge (LDC) facility at the Europeas Space Research and Technology Centre (Noordwijk, NE) during the ESA Spin Your Thesis Campaign in 2010 and 2011. The diameter of the LDC is eight metres. It has four arms that can support one gondola each with a maximum payload of 80 kg per gondola. It provides power to the equipment on board, as well as continuous video monitoring, which was used to control data acquisition during the experiments. Two independent setups, similar to the one used for the parabolic flight experiments, were positioned inside two of the four gondolas. Treatments of hypergravity consisted of spinning at 2 *g* and 5 *g* for one hour each. During the two campaigns, 10 replicated experiments were performed for each treatment.

Control experiments (N = 14) were conducted in normogravity condition (on ground, 1 *g*). All the experiments and repetitions have been performed following an identical procedure and using the same genetic material in all cases.

Data acquisition and signal processing. Data were acquired at a sampling rate of 10 KHz. Negative spikes (APs) waveforms and timestamps were obtained with a threshold based method using MC-Rack software (MCS) and processed using NeuroExplorer® (Nex Technoloigies, Littleton, MA), a multiple spike train analysis software. For the analysis, only the electrodes that were covered by the root sample were considered. In particular, according to the root anatomy, electrodes were divided in two groups covering respectively the transition zone (TZ) and the mature zone (MZ) of the root apex. On average, the electrical activity recorded by about 20 electrodes for each zone was available, and data from the 7 most active electrodes within each zone were used for the statistical analysis. Analysis of spike rate (rate of occurrence of APs) was performed for each group and repeated experiments. Cross-correlation analysis of firing events recorded from all electrodes covered by the root sample was also done. Some signals propagating from one region to the other were extracted and used for the computation of the speed of propagation. Finally, APs with similar amplitude (50–70 μV) recorded during each experiment were selected and used for shape analysis. The selection was performed in order to avoid the interference of the weakest and strongest signals on the analysis of the spike duration. Signals with small amplitude can be the result of APs generated in cells not perfectly attached to the electrode surface or, in the case of spikes with big amplitudes, as the result of the simultaneous detection of more than one AP, generated by more than one cell. Given the low frequency distribution of spikes with amplitude inferior to 50 μV or superior to 70 μV (Fig. S2), the selection did not influenced the final results since it represented the normal range of APs. The duration of each waveform was calculated using a fixed threshold (chosen as −5 μV) and the distance between x_1 and x_2, where x_1 and x_2 represent the two points of intersection of the waveform with the threshold (Fig. 6).

The data are reported as the mean ± standard error (SEM). Comparisons of the means were performed with one-way ANOVA and Tukey's post-test or with unpaired one-tailed *t*-test as non-parametric test, using GraphPad Prism 5 (GraphPad Software, San Diego, CA).

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Author contributions

S. Mancuso designed the study. E. Masi, D.C., E. Monetti, C.P., E.A., S. Mugnai and S. Mancuso conducted experiments. E. Masi, D.C. and M.C. conducted data analysis. E. Masi, F.B. and S. Mancuso wrote the paper. All the authors contributed to the discussion of results.

Additional information

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