

Single-layer graphene modulates neuronal communication and augments membrane ion currents

Niccolò Paolo Pampaloni, Martin Lottner, Michele Giugliano, Alessia Matruglio, Francesco D'Amico, Maurizio Prato, José Antonio Garrido*, Laura Ballerini*, Denis Scaini*

Supplemental results on SLG impact on neuronal networks

Large films of SLG, MLG and Au were fabricated and transferred onto bare glass coverslips. SLG and MLG samples were prepared by film transfer, either through polystyrene (PS) or polymethyl methacrylate (PMMA) supporting polymer (see Methods). PSCs measured in neurons showed however similar behaviour when carbon-based films were transferred through PMMA or PS (Supplementary Fig. 1a). This indicates that the observed effect on PSCs is independent of the carbon film transfer method.

Under our recording conditions (see Methods), spontaneous PSCs were composed of mixed events: inhibitory (GABA_A-receptor mediated) and excitatory (AMPA-glutamate receptor mediated), all recorded as inward synaptic currents. These currents are characterized by different kinetics [1,2] and were analysed offline to gain insights into the SLG regulation of synaptic activity. In particular, the decay time constant (τ) of those currents was quantified in a subset of Control and SLG neurons. We identified slow decaying PSCs ($\tau = 22.4 \pm 1$ ms in Control; $\tau = 21.2 \pm 1.2$ ms in SLG) attributed to GABA_A receptor-mediated events, and fast decaying PSCs ($\tau = 3.2 \pm 0.2$ ms in Control; $\tau = 3.4 \pm 0.3$ ms in SLG; Supplementary Fig. 1b) attributed to AMPA-receptor mediated events [2]. Fast and slow PSCs were comparably up-regulated in their frequency by SLG (Plot in Supplementary Fig. 1b).

Supplemental results on graphene cation- π interaction

Our preliminary Raman results can explain the different behaviour of SLG towards K^+ or Na^+ and the difference between SLG and MLG behaviour in ion solution. However, do not directly demonstrate differences between SLG and MLG in ion absorbance. In fact, the adsorption of ions on SLG, when on insulating substrates, may induce non-homogeneous densities of charge carriers in the monolayer that are measured by Raman spectroscopy, while such interactions are prevented, and thus not measured by Raman, when carriers are distributed in the bulk conductive substrates (the multiple graphene layers in MLG) and not confined to the SLG layer. As in a random-walk with a “sticky/viscous” wall, free potassium ions at the interface would be largely depleted, while their concentration would be unaffected in the bulk, i.e. far from the graphene surface. Therefore, at the nanoscale, at a distance compatible with realistic cell membrane proximity, a vertical K^+ spatial gradient may not be compensated entirely, as it is likely to be restricted at the interface with graphene by the tortuosity of the extracellular microenvironment, densely packed with macromolecules for cell adhesion as well as cell membranes of neighbouring cells [3]. This will translate in a slight but effective reduction of free extracellular potassium in the sub-micrometrical extracellular space confined between graphene and the overlying neuronal membrane (see Fig. 6b). The effectiveness of such a K^+ depletion in altering cell excitability is grounded by our mathematical single-cell biophysical model (Fig. 5).

Intriguingly, in our previous studies, when interfacing neurons with carbon nanotubes (CNTs, basically rolled up graphene sheets) randomly piled in dense meshes, we never observed increases in AHP, supporting the suggested mechanisms dependent on the intrinsic properties of SLG. Differently from SLG, CNTs boosted synaptogenesis and moulded the integrative abilities of cultured hippocampal

neurons, probably due to their shape, conductivity and roughness, mimicking extracellular matrix and promoting tight nano-contacts between neuronal membranes and CNTs supporting a direct electrical coupling between CNTs and neuronal membranes [1,4]. In more complex systems, CNTs scaffolds were reported also to increase and guide axonal re-growth and orientation [5,6].

The precise mechanisms for the observed effects of SLG substrates in this study are still elusive. We cannot rule out that the up-regulation of K^+ outward currents and the switch in firing patterns could be induced by other chemical or physical features of SLG, anyhow we put forward a consistent hypothesis based on the specific properties of the materials characterized by π electron-rich one plain layer of carbon atoms and we focused in particular on the specific cation- π interactions [7].

We further postulate that in the case of SLG, the more unperturbed its band structure is, the larger is its ability to deplete potassium ions at the interface with neuronal membrane (see Fig. 6c and 6d). Naturally, we cannot exclude alternative possibilities, but our results with suspended and ITO-supported SLG are consistent with our hypothesis.

Supplemental Methods for the mathematical model of a neuronal network

A Wilson-Cowan-like model, accounting for the spontaneous electrical activity observed in cultured neuronal networks, was defined and computer-simulated. It aimed at supporting the interpretation of the *in vitro* recordings and at linking (phenomenologically) single-cell properties to spontaneously emerging network activity. The model describes at the population level, the instantaneous firing rates $v_{E1}(t)$, $v_{E2}(t)$ and $v_I(t)$ of a heterogeneous ensemble of excitatory (E) and inhibitory (I)

neurons, respectively. Three populations were in fact considered (i.e. two excitatory and one inhibitory), each defined by a characteristic time scale (i.e. τ_E and τ_I), by single-cell $f-I$ curve (i.e. $\phi(I_{syn})$) and by the specific recurrent connectivity [8,9,10,11].

$$\begin{aligned}\tau_E \frac{dv_{E1}}{dt} &= -v_{E1} + \phi_{E1}(\mu_{E1}, \sigma_{E1}) \\ \tau_E \frac{dv_{E2}}{dt} &= -v_{E2} + \phi_{E2}(\mu_{E1} - g_{SFA} x_{SFA} v_{E2}, \sigma_{E2}) \\ \tau_I \frac{dv_I}{dt} &= -v_I + \phi_I(\mu_I, \sigma_I)\end{aligned}\quad (1)$$

The $f-I$ curves were described by an identical transfer function of a *leaky Integrate-and-Fire* model neuron, expressed – under the hypotheses of the diffusion approximation [12]– by an analytical formula:

$$v = \phi(\mu, \sigma) = [\tau_{arp} + R_m C_m \int_A^B \sqrt{\pi} e^{x^2} (1 + \text{erf}(x)) dx]^{-1} \quad (2)$$

where $A = \frac{V_\theta - E_m - R_m \mu}{\sigma \sqrt{R_m / C_m}}$ and $B = \frac{V_H - E_m - R_m \mu}{\sigma \sqrt{R_m / C_m}}$, with $\mu = \Delta \lambda$ and $\sigma = \Delta \sqrt{\lambda}$.

This approximation holds as long as the stochastic rate of incoming synaptic events λ is very large and the net charge associated to the unitary synaptic potential Δ is very small (i.e. while the product $\lambda \Delta$ remains finite). In the above formula only the (infinitesimal) mean μ and variance σ^2 of the incoming average synaptic inputs are considered. These statistical parameters reflected the recurrent synaptic connectivity and of external inputs, as sketched in Fig. 5a, through the size of presynaptic populations (i.e. N_{ext} , N_{E1} , N_{E2} , N_I), the probability of recurrent connectivity (i.e. c), and the average of synaptic couplings (i.e. the charge associated to each postsynaptic potential; Δ_{EE} , Δ_{EI} , Δ_{IE} , Δ_{II}) and their standard deviations (i.e. $s\Delta_{EE}$, $s\Delta_{EI}$, $s\Delta_{IE}$, $s\Delta_{II}$)

$$\mu_{E1} = N_{ext} \Delta_{ext} v_{ext} + c N_{E1} \Delta_{EE} r_{E1} v_{E1} + c N_{E2} \Delta_{EE} r_{E2} v_{E2} + c N_I \Delta_{EI} v_I \quad (3)$$

$$\begin{aligned}
\mu_{E2} &= N_{ext}\Delta_{ext}v_{ext} + c N_{E1}\Delta_{EE}r_{E1}v_{E1} + c N_{E2}\Delta_{EE}r_{E2}v_{E2} + c N_I\Delta_{EI}v_I \\
\mu_I &= N_{ext}\Delta_{ext}v_{ext} + c N_{E1}\Delta_{IE}r_{E1}v_{E1} + c N_{E2}\Delta_{IE}r_{E2}v_{E2} + c N_I\Delta_{II}v_I \\
\sigma^2_{E1} &= N_{ext}(\Delta_{ext}^2 + s\Delta_{ext}^2)v_{ext} + c N_{E1}(\Delta_{EE}^2 + s\Delta_{EE}^2)r_{E1}^2v_{E1} + c N_{E2}(\Delta_{EE}^2 \\
&\quad + s\Delta_{EE}^2)r_{E2}^2v_{E2} + c N_I(\Delta_{EI}^2 + s\Delta_{EI}^2)v_I \\
\sigma^2_{E2} &= N_{ext}(\Delta_{ext}^2 + s\Delta_{ext}^2)v_{ext} + c N_{E1}(\Delta_{EE}^2 + s\Delta_{EE}^2)r_{E1}^2v_{E1} + c N_{E2}(\Delta_{EE}^2 \\
&\quad + s\Delta_{EE}^2)r_{E2}^2v_{E2} + c N_I(\Delta_{EI}^2 + s\Delta_{EI}^2)v_I \\
\sigma^2_I &= N_{ext}(\Delta_{ext}^2 + s\Delta_{ext}^2)v_{ext} + c N_{E1}(\Delta_{IE}^2 + s\Delta_{IE}^2)r_{E1}^2v_{E1} + c N_{E2}(\Delta_{IE}^2 \\
&\quad + s\Delta_{IE}^2)r_{E2}^2v_{E2} + c N_I(\Delta_{II}^2 + s\Delta_{II}^2)v_I
\end{aligned} \tag{4}$$

Following closely [13], to approximately capture the dynamical filtering effects of AMPAr- and GABAr-mediated synapses, each presynaptic mean firing rate v in equations 4-5 was replaced by its low-passed version \hat{v} which also included the finite-size fluctuations [8]:

$$\begin{aligned}
\tau_{AMPA} \frac{d\hat{v}_{E1}}{dt} &= -\hat{v}_{E1} + \text{Poisson}[N_{E1}v_{E1}\Delta t]/(N_{E1}\Delta t) \\
\tau_{AMPA} \frac{d\hat{v}_{E2}}{dt} &= -\hat{v}_{E2} + \text{Poisson}[N_{E1}v_{E1}\Delta t]/(N_{E1}\Delta t) \\
\tau_{GABA} \frac{d\hat{v}_I}{dt} &= -\hat{v}_I + \text{Poisson}[N_{E1}v_{E1}\Delta t]/(N_{E1}\Delta t)
\end{aligned} \tag{5}$$

where, for each time t , $\text{Poisson}[x]$ indicates a new realization of a pseudo-random number, drawn from a Poisson distribution with mean x , and where Δt is the numerical integration step.

The effects of homosynaptic short-term depression at excitatory synapses and spike frequency adaptation in just one of the two excitatory populations, were finally described by three additional equations

$$\begin{aligned}
\tau_{SFA} \frac{dx_{SFA}}{dt} &= -x_{SFA} + \text{Poisson}[N_{E1}v_{E1}\Delta t]/(N_{E1}\Delta t) \\
\tau_{STD} \frac{dr_{E1}}{dt} &= 1 - r_{E1} - U r_{E1} \tau_{STD} \hat{v}_{E1}
\end{aligned} \tag{6}$$

$$\tau_{STD} \frac{dr_{E2}}{dt} = 1 - r_{E2} - U r_{E2} \tau_{STD} \hat{v}_{E2}$$

and by replacing including short-term synaptic depression, intrinsic spike frequency adaptation, and the effects to the finite size of the network [8].

Model parameter	Value
$N_{E1} + N_{E2}$	1280
N_I	400
τ_E	20 ms
τ_I	20 ms
τ_{AMPA}	10 ms
τ_{GABA}	2 ms
τ_{STD}	800 ms
τ_{SFA}	1500 ms
g_{SFA}	10 a.u.
U	0.2
$\Delta_{ext} \pm s\Delta_{ext}$	$(0.416 \pm 0.104) / C_m$ mV
$\Delta_{EE} \pm s\Delta_{EE}$	$(0.809 \pm 0.202) / C_m$ mV
$\Delta_{EI} \pm s\Delta_{EI}$	$(-0.34 \pm 0.085) / C_m$ mV
$\Delta_{IE} \pm s\Delta_{IE}$	$(1.23 \pm 0.307) / C_m$ mV
$\Delta_{II} \pm s\Delta_{II}$	$(-0.358 \pm 0.0894) / C_m$ mV
$N_{ext}v_{ext}$	1.25 kHz
c	0.25
C_m	20 pF
R_m	1 k Ω
E_m	-70 mV
V_θ	-55 mV
V_H	-70 mV
τ_{arp}	2 ms

Table 1: Numerical values employed in the simulations of Figure 5.

This model [8,9,10] reproduces *in silico* the spontaneous periodic occurrence of “bursts” of APs (Fig. 5b and 5c), synchronized across the network [10]. These spontaneous events are the network-level correlates of the PSCs, as well as of the spontaneous AP firing, observed in single-cell experiments. In the model, the ignition of each episode of spontaneous firing is a direct consequence of recurrent glutamatergic synaptic transmission (i.e. acting as a positive feedback) and of

random spontaneous release events at synaptic terminals (i.e. as in mPSCs). The termination of each spontaneous firing episode is instead determined in the model by the combined effect (i.e. acting as a negative feedback) of inhibitory synaptic connections, transient synaptic pool exhaustion underlying communication between neurons, and spike-frequency adaptation in excitatory neurons. The last mechanism does in fact slow down the repetitive (spontaneous) firing and thus decrease the synaptic net currents to downstream neurons.

Supplemental methods for conductance-based single-neuron model

A minimal model of neuronal excitability was considered by studying the classic single-compartmental conductance-based description proposed by Hodgkin and Huxley (1952). Therein, the electrical potential V across the cell membrane, satisfies the conservation of charge

$$C_m \frac{dV}{dt} = I_{Na} + I_K + I_{leak} + I_{stim} \quad (7)$$

where the sum of externally applied currents (I_{stim}), capacitive displacement currents ($C_m dV/dt$), and ionic transport currents across the membrane (I_{Na}, I_K, I_{leak}) are always balanced. The model is completely described by three additional state variables (i.e. m, h, n), expressing the voltage- and time-dependent fractions of inward and outward ionic currents, $I_{Na} = G_{Na} m^3 h (E_{Na} - V)$, $I_K = G_K n^4 (E_K - V)$, $I_{leak} = G_{leak} (E_{leak} - V)$ as a first-order kinetic process:

$$\frac{dx}{dt} = \alpha_x (1 - x) - \beta_x x, \text{ with } x \in \{m, h, n\} \quad (8)$$

Model parameters are indicated in Table 2, unless noted otherwise.

Parameter	Description	Value
C_m	<i>Specific cell capacitance</i>	0.01 $\mu\text{F}/\text{mm}^2$

E_{Na}	<i>Nernst potential for Na⁺ ions</i>	30 mV
E_K	<i>Nernst potential for K⁺ ions</i>	-75 mV
E_{leak}	<i>Nernst potential for “leak” ionic currents</i>	-80 mV
G_{Na}	<i>Maximal conductance for Na⁺ ions</i>	0.333 mS/mm ²
G_K	<i>Maximal conductance for K⁺ ions</i>	0.012 mS/mm ²
G_{leak}	<i>Maximal conductance for “leak” currents</i>	0.003 mS/mm ²
I_{stim}	<i>External DC current stimulus amplitude</i>	15 nA/mm ²
$\alpha_m(V)$	<i>Kinetic rate of Na⁺ current activation</i>	0.1 $F(0.1, V+35)$
$\beta_m(V)$	<i>Kinetic rate of Na⁺ current activation</i>	4 $\exp(-(V+60)/18)$ ms
$\alpha_h(V)$	<i>Kinetic rate of Na⁺ current inactivation</i>	0.07 $\exp(-(V+60)/20)$ ms
$\beta_h(V)$	<i>Kinetic rate of Na⁺ current inactivation</i>	$[\exp(-(V+30)/10)+1]^{-1}$ ms
$\alpha_n(V)$	<i>Kinetic rate of K⁺ current activation</i>	0.01 $F(0.1, V+50)$ ms
$\beta_n(V)$	<i>Kinetic rate of K⁺ current activation</i>	0.125 $\exp(-(V+60)/80)$ ms
$F(x,y)$	<i>Boltzmann sigmoid function</i>	$y / [1 - \exp(x * y)]$ ms
Δt	<i>Numerical solution time-step</i>	0.001 ms

Table 2 – Parameters employed in the simulations of the Hodgkin-Huxley (HH)

model. The standard HH mathematical model was numerically simulated as a minimal description of neuronal excitability to gain insight on current-clamp experimental recordings. For all the models, the source code is provided as a ModelDB entry [14] (<https://senselab.med.yale.edu/ModelDB>; accession number 230930).

The sodium inactivation

The role of sodium inactivation has been investigated in previous experiments in the cortex and spinal cord [15,16] and proposed to contribute to spike adaptation. While we cannot rule out a role for impaired Na⁺ inactivation in SLG neurons, we believe that

the strongly enhanced AHP prevents the membrane potential to reach levels at which inactivation is fully expressed to limit firing.

Figure 5e further illustrates this phenomenon, across distinct stimulus current amplitudes and over three levels of K⁺ conductances.

By definition

$$E_K = \frac{RT}{zF} \cdot \ln\left(\frac{\delta \cdot [K^+]_{EX}}{[K^+]_{IN}}\right) = E_{K \text{ control}} + \Delta \quad (9)$$

where T is the absolute temperature, R the universal gas constant, F the Faraday constant, and z = 1 K⁺ valence (i.e. $RT/zF \sim 25\text{mV}$ at room temperature), and $\Delta = RT/zF \cdot \ln(\delta)$ is a negative quantity measured in mV.

Supplemental technical details about the Raman set-up

In order to compare cation/graphene interaction in SLG and MLG in an environment close to the one where neurons were investigated, we decided to acquire the Raman spectra from samples maintained under liquid conditions. To that aim, samples were kept in a liquid cell and were covered by a thin layer of liquid solution (0.5÷1 mm in thickness; Supplementary Fig. 5a) during the whole measurement process. The experiments were performed using a Raman scattering set-up that required keeping the sample surface perpendicular to the ground. Since this procedure would not allow measuring the samples while immersed in liquids, a technical modification of the set-up has been employed. More specifically (Supplementary Fig. 5a), a 45° mirror has been included in the set-up that allowed placing the sample horizontally inside a liquid cell, enabling ease liquid exchange during experiments. Thus, SLG and MLG samples were fully cover by liquid during the entire measurements, providing more representative conditions. The use of a macro-spot (about 100 μm in

diameter) in the Raman set-up limits the occurrence of molecular fluctuations –due to a thermal effect induced by laser focusing– that may alter the measurements. Graphene Raman G-peak exhibited a change in shape and position in the presence of NaCl and KCl D₂O solutions where, in particular, the shift ascribable to K⁺ ions appears larger than the Na⁺ correlated one (Fig. 6a, insets).

This result is in agreement with theoretical studies on cation- π interaction in solvated conditions [17,18]. This specific graphene-K⁺ interaction taking place in aqueous phase is furthermore supported by additional Raman analysis of SLG substrates in dry (air) condition (see Supplementary Fig. 5b). In fact, as predicted by gas-phase simulations [17,18], in this case a stronger graphene-Na⁺ interaction (G peak shift of about $4 \pm 1 \text{ cm}^{-1}$ compared to control SLG) is detected than graphene-K⁺, confirming the key role of cations, and the specificity of potassium, in the aqueous environment and further sustaining theoretical studies [17,18].

Supplemental technical details about ion depletion in the cell/substrate cleft

Cultured neurons are characterized by a cell body displaying a “disk-like” shape with average diameters of about 10 μm (Fig. 2a and 2b and Fig. 3b). Studies of cell/electrode interfaces in culture showed typical cleft thicknesses between 40÷100 nm [19,20,21], corresponding to a cell-substrate cleft volume of about 3÷8 μm^3 .

Similar dimensions were found in our samples by SEM images of cell cross sections at membrane-substrate interface obtained by focused ion beam (FIB; Supplementary Fig. 6). At an extracellular KCl concentration of 4 mM (see Methods), $\sim 7 \div 20 \cdot 10^6$ K⁺ ions would occupy such a volume in the bulk. Taking into account, in first approximation, a 40:1 ratio between Na⁺/K⁺ cations in solution, and considering the contribution of both inner and outer hydration shells [22] to evaluate a reasonable

cross-section of interaction between K^+ and graphene, we can assume that at least $2 \cdot 10^6$ K^+ will be strongly adsorbed on the surface. This could be translated in a theoretical local depletion of potassium ions of about 10÷20% (see Fig. 6b), in accordance with what examined by our single-cell neuron model (Fig. 5e and 5f). Such a depletion profile has been inferred mesoscopically by the steady-state diffusion equation with *ad hoc* boundary conditions.

SUPPLEMENTARY REFERENCES

- [1] Cellot, G. et al, Carbon nanotube scaffolds tune synaptic strength in cultured neural circuits: novel frontiers in nanomaterial-tissue interactions. *J. Neurosci.* 31, 12945-12953 (2011).
- [2] Pampaloni, NP. et al. Sculpting neurotransmission during synaptic development by 2D nanostructured interfaces. *Nanomedicine.* S1549-9634, 30082-5 (2017).
- [3] Nicholson, C, Phillips, J. M. Ion diffusion modified by tortuosity and volume fraction in the extracellular microenvironment of the rat cerebellum. *J Physiol.* 321, 225-57 (1981).
- [4] Cellot, G. et al. Carbon nanotubes might improve neuronal performance by favouring electrical shortcuts. *Nat. Nanotechnol.* 4, 126-133 (2009).
- [5] Fabbro, A. et al. Graphene-Based Interfaces Do Not Alter Target Nerve Cells. *ACS Nano* 10, 615-623 (2016).
- [6] Usmani S, et al. 3D meshes of carbon nanotubes guide functional reconnection of segregated spinal explants. *Sci Adv.* e1600087 (2016).

- [7] French, C. R. et al. Properties of an intermediate-duration inactivation process of the voltage-gated sodium conductance in rat hippocampal CA1 neurons. *J Neurophysiol.* 115, 790-802 (2016).
- [8] Gigante, G. et al. Network Events on Multiple Space and Time Scales in Cultured Neural Networks and in a Stochastic Rate Model. *PLoS Comput Biol.* 11, e1004547 (2015).
- [9] Gambazzi, L. et al. Diminished activity-dependent brain-derived neurotrophic factor expression underlies cortical neuron microcircuit hypoconnectivity resulting from exposure to mutant huntingtin fragments. *J. Pharmacol Exp Ther.* 335, 13-22 (2010).
- [10] Giugliano, M. et al. Single-neuron discharge properties and network activity in dissociated cultures of neocortex. *J Neurophysiol.* 92, 977-996 (2004).
- [11] La Camera G, et al. The response of cortical neurons to in vivo-like input current: theory and experiment: I. Noisy inputs with stationary statistics. *Biol Cybern* 99, 279-301(2008).
- [12] Ricciardi, L. M. Diffusion Processes and Related Topics in Biology. Lecture notes in Biomathematics. Springer-Verlag (1977).
- [13] Furlan, F. et al. ERG conductance expression modulates the excitability of ventral horn GABAergic interneurons that control rhythmic oscillations in the developing mouse spinal cord. *J. Neurosci.* 27, 919-928 (2007).
- [14] Hines, M. L. et al. Model DB: A Database to Support Computational Neuroscience. *J Comput Neurosci.* 17, 7-11 (2004).
- [15] Fleidervish, IA & Gutnick, MJ. Kinetics of slow inactivation of persistent sodium current in layer V neurons of mouse neocortical slices. *J Neurophysiol.* 76, 2125-30 (1996).

- [16] Miles, GB., Dai, Y. & Brownstone, RM. Mechanisms underlying the early phase of spike frequency adaptation in mouse spinal motoneurons. *J Physiol.* 566, 519–532 (2005).
- [17] Pham T A, et al. Salt Solutions in Carbon Nanotubes: The Role of Cation- π Interactions *J. Phys. Chem. C*, 120, 7332-7338 (2016).
- [18] Williams, C D, et al. Effective Polarization in Pairwise Potentials at the Graphene–Electrolyte Interface *J. Phys. Chem. Lett.*, 8, 703-708 (2017)
- [19] Spira ME, Hai A. Multi-electrode array technologies for neuroscience and cardiology. *Nat Nanotechnol.* 8, 83-94 (2013).
- [20] Son, IH., et al. Graphene balls for lithium rechargeable batteries with fast charging and high volumetric energy densities. *Nat Comm.* 8, 1561 (2017).
- [21] Baronsky, T., et al. Cell-Substrate Dynamics of the Epithelial-To-Mesenchymal Transition. *Nano Lett.* 17, 3320-3326 (2017).
- [22] Pestova, O.N.et al. X-ray Phase Analysis of Structure of Water-Salt Systems: NaCl-H₂O and KCl-H₂O. *Russian J of Appl Chem.* 77,1066-1069 (2004)
- [23] Arosio, D. & Ratto, GM. Twenty years of fluorescence imaging of intracellular chloride. *Front Cell Neurosci.* 8, 258 (2014).
- [24] Gertler, T. S., Chan, C.S., Surmeier, D.J. Dichotomous anatomical properties of adult striatal medium spiny neurons. *J Neurosci.* 28, 10814-10824 (2008).

SUPPLEMENTARY FIGURES CAPTIONS

Supplementary Figure 1 | SLG impact on PSC frequency a, Box plots

summarizing the average PSC frequency values (left) and the average PSC amplitudes ones (right) for neurons developed on supported SLG transferred using PMMA (in blue, $n = 19$), or PS (in green, $n = 17$). SLG induces in neurons' PSCs similar effects when the carbon film is transferred through PMMA or PS. **b**, Offline differential analysis of PSC decays (τ) identifies fast and slow events (inset, average tracings from a representative SLG neuron). Bar plot summarizes the frequency of fast and slow PSCs in Controls ($n = 4$) and SLG ($n = 4$). Note that the % of distribution of fast and slow events was not changed by SLG, regardless the increased frequency (Fig. 2c). Statistically significant difference between two data sets was assessed by Student's t-test for parametric data and by Mann-Whitney for non-parametric ones.

Supplementary Figure 2 | GABA-evoked chloride fluxes in SLG and Control. a,

Snapshot of MQAE-loaded hippocampal neurons (9 DIV) in Control and SLG. Scale bars: 15 μm . When long pulses (500 ms) of pressure applications of GABA (10 mM) are delivered, efflux or influx of Cl^- are induced in the neurons, depending on their maturation, resulting in opposite changes in the Cl^- -sensitive MQAE fluorescence (middle tracings, 2 different cells). Such changes were not detected when extracellular saline solution was pressure applied via the same apparatus. In all imaged fields ($n = 20$), cells displaying opposite directions of GABA-evoked Cl^- fluxes were detected, thus indicating that immature and mature neuronal phenotypes coexist within the same network. From the bar plot at the right, summarizing the percentage of cells responding with a Cl^- influx (in blue) or efflux (in magenta) in Control and SLG, no difference in their distributions was observed in Control and

SLG cultures. **b**, NKCC1, the most abundant co-transporter membrane protein determining intracellular chloride levels [23], is expressed on hippocampal neurons in Control and SLG. Confocal images of neuronal cultures (9 DIV) in Control and SLG demonstrate NKCC1 (marked in green) in class III β -tubulin positive cells (marked in red). Merged images are displayed for clarification. Scale bars: 30 μ m. The histograms summarize NKCC1 volume normalized to class III β -tubulin volume in the two conditions revealing comparable NKCC1 expression in Control and SLG class III β -tubulin-positive neurons (n = 20 field each, P = 0.37). Statistically significant difference between two parametric data sets was assessed by Student's t-test.

Supplementary Figure 3 | Firing patterns evoked in Control and SLG neurons by near-threshold current steps. a. APs were elicited by 200 ms current steps of 40 pA (blue traces) and 60 pA (orange traces) amplitudes in control (n = 5) and SLG (n = 5) neurons kept at -60 mV membrane potential. Plot in **b.** shows that SLG neurons usually fire more APs at near-threshold (60 pA) current injections with respect to Controls. The estimated rheobase current values [24] did not differ in these two groups of neurons (56.5 ± 12.9 pA and 60.5 ± 12.8 pA, control and SLG neurons respectively).

Supplementary Figure 4 | Robustness of the mathematical model. The mathematical model of neuronal excitability considered in the paper was systematically explored testing the robustness of our conclusions. The space of parameters, represented by the ionic maximal conductances for sodium and potassium channels, was considered and subdivided in regions with transient (black)

and sustained (white) firing responses. Increasing inward currents by the value of sodium maximal conductances obviously increases excitability: i.e. moving *from left to right while in the dark brings to the white peninsula*. However, there is a wide portion of the plane where increasing outward currents by the value of potassium maximal conductances also increases excitability: i.e. moving *from bottom to top while in the dark brings to the peninsula*. The transition of neuronal phenotype, observed comparing control and SLG conditions (Fig. 4c), resembles more the latter than the former case. Parameters: G_K in [0.02; 0.11] mS/mm², G_{Na} in [0.01; 0.91] mS/mm², I_{stim} 20 nA/mm².

Supplementary Figure 5 | Raman set-up and air measurements. **a**, Schematic representation of the technical modifications adopted in the standard backscattering Raman set-up to allow measurements taking advantage of a liquid cells to obtain graphene Raman spectra in genuine liquid conditions. **b**, SLG Raman spectra of G band in dry condition (e.g. after samples were carefully rinsed with D₂O and let dry in a N₂ box for 1 hour). Control condition (air, in black) is compared with spectra of graphene previously immersed in 4 mM D₂O of KCl (in red) and 150 mM solution of NaCl (in green). Note that, differently from the wet experiments depicted in Figure 6a, NaCl induce now in SLG a larger shift in G-Peak position than KCl (see insets).

Supplementary Figure 6 | Cell-substrate cleft dimension experimental validation. **a**, Representative SEM image of a neuronal cell developed above a glass substrate. The white arrow indicates the cell region where focused ion beam (FIB) was used to obtain a cell cross section. **b**, SEM magnification of the milled cell portion. The image points out the presence of a gap between the cell process and

the glass substrate (brighter region of electron accumulation) of about hundreds of nm, presumably filled with extracellular matrix and solution. **c**, A cartoon pointing out the different components visible in the cross section. Interestingly, the gap appears variable in its extent: larger centrally and smaller at the edges. **d**, Representative SEM image of a neuronal cell developed above SLG. The arrow indicates the milled cell region. **e**, SEM image pointing out the presence of a similar gap between the cell process and the substrate of nearly one hundred of nm. The thin single layer of graphene, a fraction of nm in thickness, is not detectable but the underneath supporting glass is clearly visible (brighter region of electron accumulation). The cell/substrate distance appears smaller than on control glass. **f**, A cartoon pointing out the different components visible in the cross section. Lateral cellular or extracellular patches seem partially closing the gap. Scale bars are 5 μm in a and d; 200 nm in b and e. Note that in both conditions (Control and SLG) we detected variable ranges of gap dimensions, in accordance with the values reported in the literature [21].