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Thermally reduced graphene is a permissive material for neurons and astrocytes and *de novo* neurogenesis in the adult olfactory bulb *in vivo*

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ABSTRACT

Graphene and graphene-based nanomaterials (GBNs) are being investigated as potential substrates for the growth of neural stem cells (NSCs), neurons and glia in cell culture models. In contrast, reports testing the effects of graphene directly with adult neural cells *in vivo* are missing. Here we studied the biocompatibility of thermally reduced graphene (TRG) with neurons and glia, as well as with the generation of new neurons in the adult brain *in vivo*. TRG injected in the brain together with a retroviral vector expressing GFP to label dividing progenitor cells in the core of the adult olfactory bulb (OB) did not alter *de novo* neurogenesis, neuronal and astrocyte survival nor did it produce a microglial response. These findings indicate that TRG may be a biocompatible material with neuronal and glial cells *in vivo* and support its use in studies of brain repair and function.

1. Introduction

The unique mechanical and chemical properties of graphene and its high electrical conductivity make this material potentially suitable for a range of applications in tissue engineering, regenerative medicine and drug delivery [1–4]. Graphene is the basic building block of all graphitic allotropes, which is a two-dimensional, one-atom-thick carbon sheet with a planar honeycomb lattice. Single layer graphene was firstly isolated using a micromechanical cleavage of graphite [1]. However, this technique is a time consuming approach, yielding small quantities and hindering the effective and full exploitation of this material. Therefore, chemical procedures have been pursued to obtain large-scale synthesis. The most widely used method to produce GBNs from graphite is by its complete oxidation to graphite oxide (GO) and subsequent thermal reduction of the acid groups. This reduction process is not complete and some remaining epoxy, hydroxyl and carboxyl groups are present on the surface of graphene, resulting in thermally reduced graphene (TRG) or functionalized graphene sheets that can be readily modified with desired molecules [5–9].

A number of recent works have reported that graphene and GBNs can serve as substrates for NSC differentiation, neuronal and oligodendrocyte growth and the function of neural circuits *in vitro* [10–22]. However, reports testing the biocompatibility of graphene with adult neural cells *in vivo*, including its effects on adult neurogenesis have yet to be published. Here, we have investigated the effects of TRG produced by rapid thermal expansion of GO (see Methods) on cell survival and neuron generation in the adult mouse OB, a brain region where the formation of new neurons continues in the adult [23,24]. The response of microglial cells to the injected TRG was also studied as a mean to explore the occurrence of brain inflammation.

Our findings indicate that TRG is a permissive material that produces no deleterious effect on neurons and astrocytes. The generation of new neurons from progenitor cells is observed in the presence of TRG *in vivo* with no signs of an inflammatory response indicating that this material may be biocompatible with neural cells for studies of brain function and repair.

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2. Materials and methods

2.1. Animals

All animal care and handling was carried out in accordance with European Union guidelines (directive 2010/63/EU) and Spanish legislation (Law 32/2007 and RD 53/2013), and the protocols were approved by the Ethical Committee of the Consejo Superior de Investigaciones Científicas (CSIC) and Comunidad de Madrid. Food and water were administered *ad libitum* and environmental conditions were strictly controlled: 12 h light/dark cycle, temperature 22 °C, and humidity 44%. All efforts were made to ameliorate the suffering of the animals.

2.2. Preparation of TRG

As reported previously [7,25], GO was produced from natural graphite powder (universal grade, 200 mesh, 99.9995%) according to the Brödie method. TRG was then formed by the rapid thermal expansion of GO at 1000 °C in an Argon atmosphere. The resulting material consists of partially oxygenated or thermally reduced graphene (TRG, as mentioned above). Next, this material was attached on glass coverslips previously coated with 0.5 wt.-% polyethyleneimine to be analysed by 5 μm × 5 μm tapping-mode atomic force microscopy (AFM) (Fig. 1A–C). Transmission electron microscopy (TEM) images (Fig. 1D) were taken on a Philips Tecnai 20 TEM, operated at 200 kV. The TRG was suspended in a DMF solution and deposited on standard holey carbon copper grids using the drop cast method and loaded into the microscope.

The electrical conductivity measurement was performed using the four-probe method to eliminate the effect of contact resistance [26]. Samples were cut with a length of 5 mm with a cross-sectional area of 10.28 mm^2. A Keithley 6512 digital multimeter equipped with a YEW2553DC current–voltage standard was used to measure the current–voltage characteristics (I–V characteristics) of the samples at room temperature. Then, the resistivity (ρ) was calculated as follows: $\rho = R \times A/L$ where R is the electrical resistance of a sample of the material, L is the length of the piece of material and A is the cross-sectional area of the sample. The conductivity (σ) was calculated as 1/ρ and expressed in Siemens (S)/m.

2.3. Injection of TRG and retroviral vectors expressing EGFP in the adult mouse brain

2.3.1. Dispersion and injection of TRG in PBS

Once powder TRG was obtained, it was dispersed in PBS at a concentration of 0.004 μg/μl. This was the maximum dose of TRG that could be uniformly dispersed and stereotaxically injected. The suspension was sonicated to achieve a uniform distribution of the TRG. Four μl TRG were injected into the core of the OB (Fig. 1E and G) in order to evaluate the effect of TRG on the populations of neurons and astrocytes resident in this region and on microglia. The PBS for the control side injections (4 μl) was maintained under the same conditions during the preparation of the TRG (Fig. 1E, top).

2.3.2. Preparation of retroviral particles and injection of TRG together with a pRV-EGFP vector

A retroviral vector (pRV) expressing the Enhanced Green Fluorescent Protein (pRV-EGFP) was used to mark proliferative cells and their progeny *in vivo* [27] in the presence of TRG (Fig. 1F). Particles were obtained by transfecting the retroviral plasmids into 1F8 cells, a monoclonal cell line derived from a stock of 293GPG cells [28]. The supernatant containing the retroviral particles was then concentrated by ultracentrifugation and the resulting titers were in the range of 10^{12}–10^{13} colony forming units (cfu)/ml. Then, the TRG

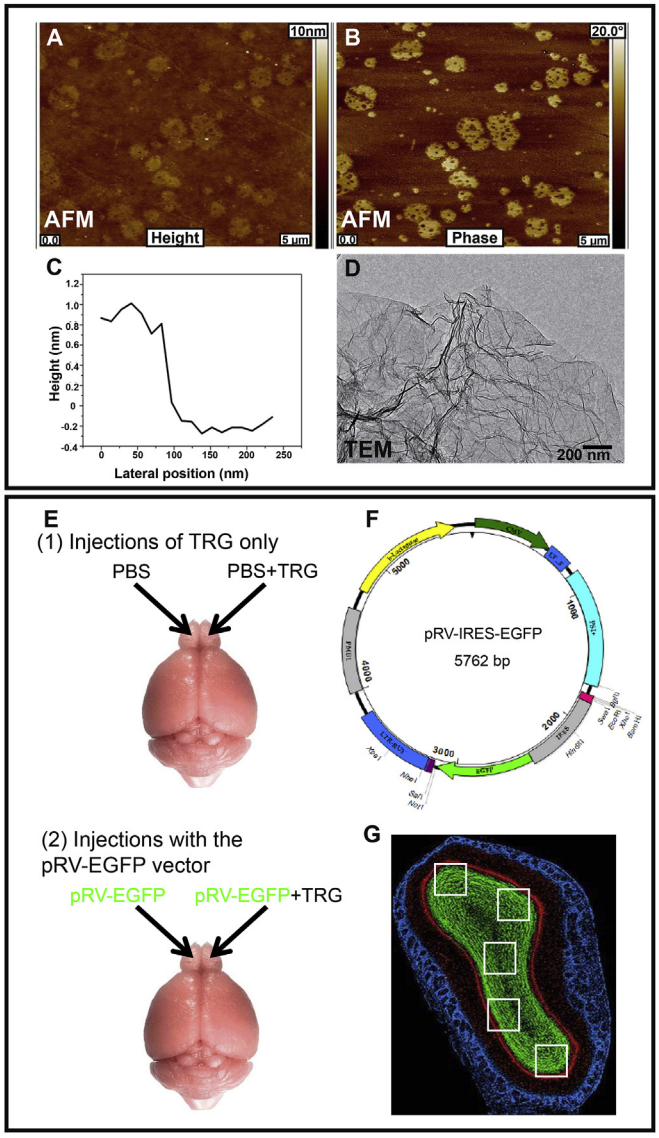

Fig. 1. The characterization of thermally reduced graphene (TRG) and experimental design of stereotaxic injections in the adult brain. The AFM images (A, B) show the size of the graphene layers and the height profile, which does not exceed 1.2 nm, making it few-layer graphene (C). The TEM image shows the characteristic wrinkle structure of the material (D). Scheme (E) summarizes the different sets of injections performed into the olfactory bulb (OB) of the adult brain and (F) represents the structure of the pRV-EGFP retroviral vector. Scheme (G) illustrates how the cell counting was performed on histological sections. Squares mark the five areas counted for every section for later statistical analyses. AFM: Atomic force microscopy; PBS: Phosphate Buffer Saline; bp: base pairs; TEM: Transmission Electron Microscopy; TRG: Thermally reduced graphene.

dispersed in PBS (2 μl) together with the pRV-EGFP vector (2 μl) were injected to study the effect of TRG on neuron generation from the dividing progenitors in the subependymal zone/rostral migratory stream (SEZ/RMS) of the OB core, as well as the effect on the resident neuron and astrocyte populations and microglia. The control side received injections of pRV-EGFP vector (2 μl) in PBS (2 μl) (Fig. 1E, bottom).

2.3.3. Stereotaxic surgery procedure

The mice (9-week-old wild-type C57BL/6N) were operated using a digital stereotaxic instrument and were maintained under an inhalatory mix of anesthesia (Isoflurane) and oxygen at a rate of 0.8–1 Litre/min during the whole operation. Once the mouse was