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A unique method for the isolation of nasal olfactory stem cells in living rats

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Abstract Stem cells are attractive tools to develop new therapeutic strategies for a variety of disorders. While ethical and technical issues, associated with embryonic, fetal and neural stem cells, limit the translation to clinical applications, the nasal stem cells identified in the human olfactory mucosa stand as a promising candidate for stem cell-based therapies. Located in the back of the nose, this multipotent stem cell type is readily accessible in humans, a feature that makes these cells highly suitable for the development of autologous cell-based therapies. However, preclinical studies based on autologous transplantation of rodent olfactory stem cells are impeded because of the narrow opening of the nasal cavity. In this study, we report the development of a unique method permitting to quickly and safely biopsy olfactory mucosa in rats. Using this newly developed technique, rat stem cells expressing the stem cell marker Nestin were successfully isolated without requiring the sacrifice of the donor animal. As an evidence of the self-renewal capacity of the isolated cells, several millions of rat cells were amplified from a single biopsy within four weeks. Using an olfactory discrimination test, we additionally showed that this novel biopsy method does not affect the sense of smell and the learning and memory abilities of the operated animals. This study describes for the first time a methodology allowing the derivation of rat nasal cells in a way that is suitable for studying the effects of autologous transplantation of any cell type present in the olfactory mucosa in a wide variety of rat models.

Introduction

The use of stem cells for clinical applications represents a scientific challenge prompting innovative strategies and raising great hopes. To date, however, their use in clinic is rather limited to a few applications. Even though some bone, skin as well as corneal diseases or injuries can be treated with grafting of related tissues for which the success relies on stem cells present in the graft, hematopoietic stem cells remain the only stem cell type routinely used in the clinic. Indeed, hematopoietic stem cell transplantation allows for the treatment of diseases and conditions of the blood and immune system. Nevertheless, the plastic properties and the self-renewal capacities characterizing a stem cell raise ever

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growing hopes for the development of novel therapeutic strategies and have been proposed as a potential means of treatment for a variety of disorders (Brignier and Gewirtz, 2010; George, 2010; Kim and de Vellis, 2009; Lawall et al., 2010; Lindvall and Kokaia, 2006; Ronaghi et al., 2010). Despite the accumulation of data in favor of the utilization of stem cells and/or their derivatives, the translation from the bench to the bedside remains difficult to achieve. Thus, several problems such as ethical issues, cell availability, graft rejection and the risk of tumor formation can potentially be associated with their use (Boyd et al., 2012; Cantz and Martin, 2010; Li et al., 2008; Somoza and Rubio, 2012), which considerably curb their application to the clinic. Accordingly, the search for both stem cells and novel models of study allowing to bypass these constraints could contribute to speed up the long process leading to the development of new stem cell-based strategies. Along this line, the development of new methods involving autologous transplantations, when applicable, would represent a major advantage for the patients' safety and avoid several complications, placing this kind of research as one of the priorities. Nevertheless, animal models allowing the assessment of the effects induced by autologous stem cell transplantations are limited and often replaced by syngeneic transplantation instead.

Among the potential stem cell candidates for therapeutic development, we have highlighted the existence of a promising multipotent contender residing within the nasal olfactory mucosa (Delorme et al., 2010; Murrell et al., 2005; Tome et al., 2009), a peripheral and permanently self-renewing nervous tissue (Lindsay et al., 2010). This cell type has been characterized as a member of the mesenchymal stem cell superfamily displaying multilineage differentiation properties and a high proliferation rate in vitro (Delorme et al., 2010; Murrell et al., 2005; Tome et al., 2009). Of note, we coined the name "olfactory ecto-mesenchymal stem cells" (OE-MSCs) to define this new stem cell type (Delorme et al., 2010). Importantly, these cells present the advantage to be of ease access in human (Girard et al., 2011), which support their potential usefulness for autologous transplantation. In rodents, OE-MSCs have been successfully used in different models including myocardial infarct (McDonald et al., 2010), spinal cord trauma (Toft et al., 2012; Xiao et al., 2005, 2007), cochlear damage (Pandit et al., 2011) and Parkinson's disease (Murrell et al., 2008) as well as in a mouse model that mimics effects of ischemic/hypoxic injury in the hippocampus (Nivet et al., 2011).

In the present study, we focused our attention on olfactory lamina propria-derived stem cells. However, it can be pointed out that this tissue also harbors another cell type of great interest for the regenerative medicine, namely the olfactory ensheathing cells (Feron et al., 2005; Mackay-Sim et al., 2008).

Despite great promises raised by these different studies and the use of stem cells and/or ensheathing cells for clinical purposes, one major hurdle resides in the lack of a method allowing the isolation of these cells in living animals for testing the effects of autologous transplantations. Up to date, all data that have been accumulated rely on the use of syngeneic models (Bianco et al., 2004; Lu et al., 2001, 2002; McDonald et al., 2010; Toft et al., 2012) or xenotransplantations of human stem cells in rodent models (Murrell et al., 2008; Nivet et al., 2011; Pandit et al., 2011; Xiao et al., 2005, 2007) requiring in most cases the use of an immunosuppressant. Accordingly, the establishment of a method to harvest stem or ensheathing cells in an animal that could be at the same time the donor and the receiver of its own cells may represent a major advance to further test the full potential of these cells while excluding complications/side effects associated to the use of alternative methods of transplantation. While human olfactory mucosa is readily accessible in humans, allowing autologous transplantations, the narrow opening of the rodent nasal cavities can be seen as an impassable hurdle, preventing the possibility of cell autologous grafts in mice or rats. To overcome this problem, we developed a unique technique of olfactory mucosa excision allowing autologous grafts of nasal cells in rats. Biopsy collection, rodent nasal stem cell characterization and amplification as well as the consequence of the excision on the sense of smell and cognitive abilities of the donor animals are reported in this study.

Materials and methods

Rats

Ten-week-old male Sprague Dawley rats (n = 50; Charles River), at the beginning of the experiment, were used. All animals were housed in individual cages and maintained on a 12-hour light/12-hour dark cycle at a constant temperature (22 +/− 1 °C). Food and water were provided ad libitum except when tested for an olfactory associative discrimination task. Anesthesia and surgical procedures were performed according to the European law on Animal Care Guidelines, and the Animal Care Committee of Aix-Marseille University approved our protocols.

Excision of rat olfactory mucosa

Rat nasal olfactory mucosae were obtained by biopsies under anesthesia (Sodium pentobarbital, Nembutal, 60 mg/kg, ip) and under analgesia (buprenorphine hydrochloride, 0.03 mg/kg, paracetamol 4 mg/kg, ip). In a first set of experiments (n = 16), we tried to obtain pieces of olfactory mucosa by creating a 2 mm long and 1 mm wide lateral aperture (right or left) along the rostro-caudal axis, allowing a direct access to the olfactory mucosa. Then, in order to improve the success rate, we performed, in 34 rats, a 1 mm long and 2 mm wide medial aperture (on top the septum), allowing an excision on both sides of the nasal cavity (Fig. 1). More precisely, the head was first inserted in a stereotactic frame. After resection of the tissues covering the nasal and frontal bones at the level of the ocular globes, the window was drilled in the skull just anterior to the cribriform plate, 3.5 mm posterior to the nasofrontal suture and 9 mm anterior to the bregma, according to the rat brain atlas of Paxinos and Watson (2005). Using a "Hartmann Alligator Micro-Forceps" (model MCO13B, Microfrance, Medtronic), a 1 square millimeter biopsy was excised. This piece of olfactory mucosa was immediately transferred using a sterile needle into a sterile 2 mL tube filled with 37 °C DMEM/HAM's F12 culture medium (CM) supplemented with 10% Fetal Bovine Serum (FBS), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Life Technologies). In order to exclude all risk of contamination, antibiotic concentrations can be doubled at 200 units/mL of penicillin and 200 μg/mL of streptomycin and 1.25 μg/mL of

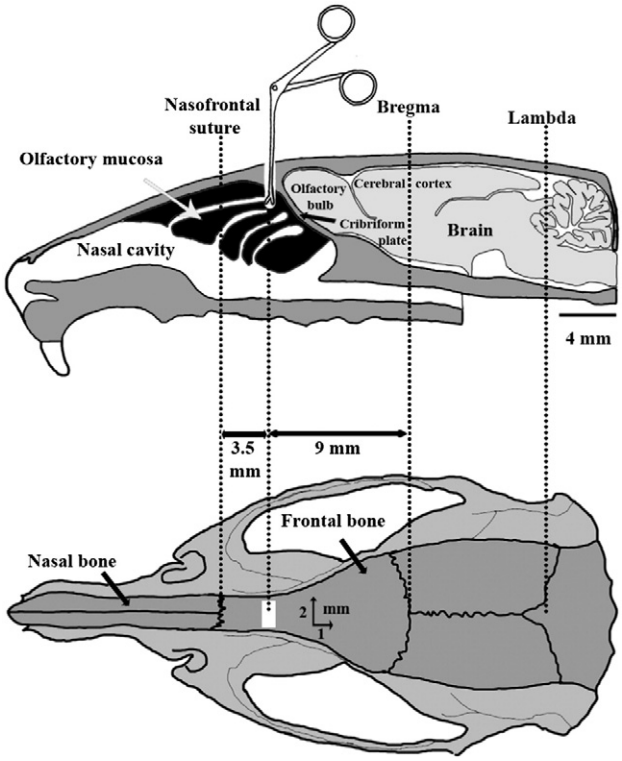

Figure 1 Biopsy of olfactory mucosa in adult rat. Rat muzzle drawings indicate the stereotaxic coordinates of the olfactory mucosa excision. As shown from the midsagittal and the dorsal views, the skull was drilled just anterior to the cribriform plate, 3.5 mm posterior to the nasofrontal suture and 9 mm anterior to the bregma. The olfactory mucosa was excised using a Hartmann-type micro-forceps.
Modified from Paxinos (2004) and Paxinos and Watson (2005).

amphotericin B (Fungizone, Sigma-Aldrich) can be added to the CM and maintained during the first week of culture. Then, the tube was tipped upside down to make sure that the biopsy was immersed in the culture medium.

Isolation and expansion of stem cells

The biopsies were processed to isolate rat nasal stem cells according to previously described methods with modifications (Feron et al., 2013; Girard et al., 2011). Two different culture methods were assessed with the biopsies obtained from lateral excisions (n = 16). For half of the group (n = 8), the collected pieces of olfactory mucosa were washed in the culture medium and immediately plated on 2 cm^2 plastic dishes. The other half of the biopsies was enzymatically and mechanically dissociated. Briefly, after washing in the culture medium, the biopsies were immersed in 0.5 mL serum-free culture medium containing 0.25% collagenase 1A (Life Technologies), during 10 min at 37 °C. Dissociation of the tissue was achieved by mechanical trituration, with the help of a pipette, at three different times: at the start, in the middle and at the end of the incubation period. Collagenase activity was inhibited by adding 5 mL of calcium-free and magnesium-free PBS (Phosphate-Buffered Saline, Life Technologies). The tube was then centrifuged at 300 ×*g*, for 5 min. The cell pellet was resuspended in the previously described culture medium (CM) and then plated on 2 cm^2 plastic dishes. The culture medium was renewed every 2 to 3 days. About one week later, proliferation of rapidly dividing cells was observed in the culture dish. When confluence was reached, the cells were detached using a trypsin-EDTA solution (0.05%, Life Technologies), centrifuged at 300 ×*g*, for 5 min and replated in a dish with a two fold increased surface area (i.e. 4 cm^2) in the previously described culture medium (CM). Similarly, at each passage, a 1:2 cell split ratio was used. Then, the medial collection of biopsies from 34 supplementary rats was performed in the following chronological order. A group of 10 rats was halved and the two above-described culture methods were assessed. Then, all remaining biopsies (n = 24) were cultivated after enzymatic and mechanic dissociations. A test on the effect of poly-L-lysine coating was performed using 8 out of 24 biopsies. They were cultivated into culture dishes pretreated or not with poly-L-lysine, for at least 2 h, at the concentration of 5 $\mu g/cm^2$ (Sigma-Aldrich). Since the poly-L-lysine coating provided a more robust cell adhesion and proliferation, the culture of the 16 remaining biopsies was performed using this coating at every passage.

Generation of spheres

In order to generate stem cell spheres, trypsinized cells were re-plated at a density of 30,000 cells/cm^2 into culture dishes pretreated with poly-L-lysine (5 $\mu g/cm^2$) and fed with serum-free DMEM/HAM'S F12 medium, supplemented with insulin, transferrin, selenium (ITS-X, 1%, Life Technologies), Epidermal Growth Factor (EGF, 50 ng/mL, R&D systems), and Fibroblast Growth Factor 2, (FGF2, 50 ng/mL, R&D systems). This culture medium was changed every other day.

Immunostaining

Immunocytochemistry was carried out to analyze Nestin and S100A4 expression. Cells were plated on glass coverslips at a density of 15,000 cells/cm^2 in the previously described culture medium (CM) containing 10% FBS for 48 h. Cells were then fixed, using a paraformaldehyde solution (4%, Antigenfix, MM France) during 15 min, and incubated with a blocking solution (3% Bovine Serum Albumin and 0.1% Triton X-100 in PBS), during 90 min at room temperature. The following primary antibodies were used: mouse monoclonal anti-Nestin (1/250, Abcys), rabbit monoclonal anti-S100A4 (1/200, Abcam). Cultures were incubated with primary antibodies, diluted in the blocking solution overnight at 4 °C, washed 3 times in PBS and then incubated with the appropriate conjugated secondary antibody, during 90 min, at room temperature in dark conditions. The following secondary antibodies were used: goat anti-mouse IgG conjugated with AlexaFluor 488 (1/500, Jackson ImmunoResearch) and goat anti-rabbit IgG conjugated with AlexaFluor 594 (1/500, Jackson ImmunoResearch). After several washes in PBS, cultures were counterstained with 0.5 μg/mL Hoechst blue (33258, Sigma-Aldrich) for 30 min and

mounted with an anti-fading medium (ProLong® Gold, Life Technologies).

Olfactory associative discrimination task

Apparatus and training procedure

Three weeks post-surgery, the potential effect of the biopsy on the sense of smell and on associative memory performance of excised rats was tested using an olfactory associative discrimination task developed in our laboratory (Jacquet et al., 2013; Marchetti et al., 2008; Roman et al., 1987). The olfactory training apparatus was a rectangular box made of wire mesh (30 cm × 30 cm × 50 cm). A conical odor port (1.5 cm in diameter, 0.5 cm above the floor) was drilled horizontally through a triangular wedge of Plexiglas, mounted in one corner of the cage. A circular (1 cm diameter) water port in the shape of a well was placed directly above the odor port. Responses to the odor presentation were monitored by a photoelectric circuit. Two flashlight bulbs which could be turned on and off when required by the testing conditions, were placed outside the cage, one on each side of the odor and water ports, 10 cm above the floor. Individual odors were delivered by forcing clean air (0.7 bars) through one of two 1 L Erlenmeyer flasks that contained 500 mL of water mixed with 0.2% of chemical odorants (jasmine vs. strawberry) from Sigma Inc. Non-odorized air, between each odor presentation, was delivered by sending air through a flask that contained only water. Odorized and clean air streams were passed individually through tubes that were put through the back of the sound-attenuating chamber and attached to the odor port. Water was delivered using a gravity-fed system, and passed through a valve which, when opened, allowed 0.1 mL of water to be released into the water port.

All experiments were conducted simultaneously in four olfactory cages to ensure that, in each experiment, representatives from each group were trained at the same time, and thus under identical conditions. Animals were trained to make two odor-reward associations. Each odor had to be associated with a specific reward, one arbitrarily designated as positive and the other as negative. One session was made of 60 trials using a successive "Go" or "No–Go" paradigm. Individual trials (S+ or S−) were run in a quasi-random fashion (no more than 3 consecutive trials with the same valence). When the odor (S+) was delivered into the cage, if rat responded by going to the water port a reward of 0.1 mL of water was obtained. The same response to delivery of the other odor (S−) resulted in no water and activation of an error light. The maximum duration of odor presentation (S+ or S−) was 10 s. Correct responses, therefore, corresponded to "Go" before the 10 second presentation for one odor had elapsed (S+) and "No–Go" to the other (S−). After having responded or not to the odor presentation (the trial), a fixed inter-trial interval (ITI) of 15 s with clean air started. If a response was given during the last second of the ITI, the next trial was delayed by 10 s and delayed by additional 10 second fractions as long as the rat was still present during the last second of each fraction in the corner where the reward was delivered. Consequently, the duration between two trials lasted more than 15 s and constituted the cumulative time to the minimum ITI. Animals were tested every day between 08:00 am and 02:00 pm. Then, all animals were trained to make associations with the odor pair for five sessions at a pace of one session per day.

Data analysis

Correct responses were characterized as "Go" for the positive odor and "No–Go" for the negative one. Incorrect responses were "Go" for the negative odor and "No–Go" for the positive one. The number of correct responses for both positive and negative odors was expressed as a percentage of the total number of odor presentations (60 per session), thereby providing a global estimate of performance for all groups (global memory). Training continued until a criterion of 80 ± 5% correct responses was reached by the control group, i.e. non-operated. This level of performance is required to ensure that all animals have learned both associations. Latencies for positive (S+) and negative (S−) odors were recorded and represented the time elapsed between the beginning of a trial and its end (10 s), when the rat responded to the odor (reference memory, which is presumed to correspond in some aspects to declarative memory in humans). When a rat did not respond, a latency of 10 s was scored. The median cumulative time (accounting for the efficiency of some aspects of procedural memory) was the number of seconds above the fixed 15 second ITI, divided by the number of ITIs which, in this experiment, amounted 59. All behavioral events were controlled and recorded by a computer.

Statistical analysis

Statistical analyses were performed with SPSS/PC +statistics 11.0 software marketed by SPSS, Inc. All data are presented as means ± SEM. Performance was analyzed using a repeated-measure MANOVA. Then, subsequent ANOVAs for each session were computed when necessary. The threshold for significance was set at $p \leq 0.05$.

Results and discussion

We first decided to evaluate the possibility to biopsy olfactory mucosa in anesthetized adult rats in order to avoid the sacrifice of the donor animal prior to harvest the tissue of interest. To this end, we sought for a new methodology involving the creation of a new route of access to the tissue. Accordingly, we studied the rat anatomy and defined stereotactic coordinates allowing the drilling of a hole that is safe for the animal and provides an access to the olfactory tissue. We found that a small opening of about 2 mm^2 in the frontal bone (see methods for the exact coordinates) was sufficient to reach the olfactory mucosa located within the posterior nasal cavity immediately anterior to the cribriform plate (Fig. 1). Importantly, by introducing fine micro-forceps through the drilled window, we demonstrated the possibility to collect small pieces of olfactory mucosa. Noticeably, biopsied animals were able to recover immediately after the surgery without showing any signs of pain or distress and none of them (n = 50) died after surgery. However only the medial excision method (n = 34) provided a successful and healthy culture of olfactory stem cells. When compared with the technique using non-dissociated olfactory mucosa (n = 5), the protocol based on enzymatic and mechanical

dissociation (n = 5) was more successful. However, in some cases, we observed morphological modifications of the isolated cells, associated to an increased cell death and a reduced proliferation rate. To avoid this phenomenon and obtain a larger number of stem cells, the effect of a poly-L-lysine coating was assessed on 8 others biopsies. We observed that the use of poly-L-lysine, at every passage, had a significant positive effect on the health and the proliferation of these cultures. We also observed that cell plating density should be over or equal to 50%, especially during the first steps of amplification, to get a growing culture. With these conditions, it is possible, in less than four weeks, to obtain a stem cell population large enough to perform an autologous graft. Once all culture conditions adjusted, the final experiment was performed using 8 rats in which the olfactory mucosa was collected using a medial aperture. The olfactory cells were isolated by a dual enzymatic and mechanic dissociation and cultivated in poly-L-lysine-coated dishes. Eight rats (control group) underwent a sham operation without biopsy excision.

To validate that the harvested tissue was the targeted one, we evaluated whether it was possible to isolate stem cells. Following a previously described procedure allowing the isolation of rat olfactory stem cells (Girard et al., 2011), we successfully derived cells exhibiting a mesenchymal stem cell like morphology (Fig. 2A). As expected, cells were able to generate spheres, when cultivated in the appropriate culture conditions (Fig. 2B) and expressed Nestin, a protein which is one of the most prominent hallmarks of stemness (Fig. 2C). Moreover, purified rat cells were immuno-positive for S100A4, a protein produced at high level in human nasal olfactory stem cells (Fig. 2D), as previously reported (Delorme et al., 2010). Human olfactory stem cells have been previously described as a highly proliferative cell type (Delorme et al., 2010) and this characteristic was confirmed in our rat cell cultures. Indeed, even though we established our cultures from very small pieces of tissue, several millions of cells were derived in less than a month time. We confirm here that rat olfactory stem cells can be successfully amplified by successive cell passages.

Our study being mainly focused on a new surgical technique, we did not assess the purity of our olfactory stem cell cultures. Nonetheless, we can rule out a putative contamination with respiratory stem cells since the biopsy was excised from the very posterior part of the nasal cavity where only olfactory mucosa lies on the cartilage (Astic and Saucier, 1986). It is more difficult to ascertain that other contaminating cell types, especially ensheathing cells, were not present in the dishes. It is however unlikely since spheres were generated and all cells were immuno-positive for Nestin and S100A4, two recognized markers for olfactory lamina propria-derived stem cells (Delorme et al., 2010). In addition, cells were grown up to passage 20 and no study has so far reported that ensheathing cells exhibit such a high proliferation ability and are able to generate spheres.

The self-renewal capacity of the peripheral olfactory tissue (Chehrehasa et al., 2012; Graziadei and Metcalf, 1971; Suzuki et al., 1998) and the limited size of the harvested tissue (2 mm^2) compared to the overall (675 mm^2) surface of olfactory mucosa (Gross et al., 1982) explain why the sense of smell is not impaired. To confirm the non-deleterious effect of biopsy excision on rat olfaction and learning/memory capacities, we used an olfactory associative discrimination task, three weeks after the surgery.

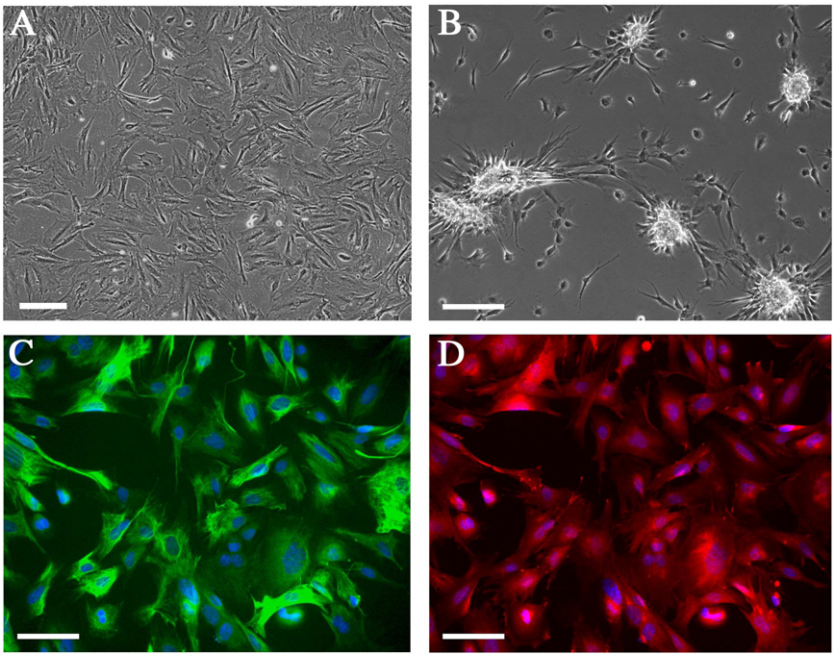

Figure 2 In vitro analysis of olfactory stem cells from adult rat olfactory mucosa. Stem cells, obtained with the reported method, exhibit a mesenchymal morphology (A). Two key features of stemness, sphere formation and Nestin expression, were evaluated (B and C). When grown in appropriate culture conditions, stem cells give rise to spheres (B) and most of them highly expressed Nestin (in green, C). These cells also express a high level of S100A4, a marker for human nasal olfactory stem cells (in red, D). Each image is representative of three independent cultures from three different rats. Scale bars, 200 μm.

Analyses of the percentage of correct responses (Fig. 3A) showed that both excised and control groups improved their overall performance similarly across the five sessions (MANOVA, Group × Session interaction: [F(4,56) = 0.15, NS]) and the overall Group effect was not significant [F(1,14) = 0.14, NS]. The cumulative time (Fig. 3B) decreased across sessions for both groups. The Group × Session interaction was not significant [F(4,56) = 0.29, NS] and no significant difference was observed between these two groups along the five successive sessions [F(1,14) = 0.41, NS]. Training performance analyzed in terms of S+ and S− latencies (Fig. 3C) showed no impairment on odor detection and memory performance in the excised group. Indeed, similarly to the Controls, excised rats were able to make correct associations on S+ and S− stimuli. In the control group, correct associations were observed from the fourth session onwards [$F(1,15) \geq 37.74$; $p < 0.001$] and the excised rats since the third session [$F(1,15) \geq 8.11$; $p < 0.05$]. The excised and the control groups showed a continuous decrease in latencies of response to S+ stimuli throughout the training session while for S− stimuli a decrease was observed during sessions 2 and 3, prior to withhold their responses (and therefore, started to exhibit correct behavior). Consequently, no significant difference between groups was observed across sessions on S + [F(4,56) = 1.27; NS] and S− stimuli [F(4,56) = 0.66; NS]. These behavioral results demonstrate that olfactory biopsies do not affect the sense of smell since operated rats are able to keep discriminating the odors, as demonstrated by their capacity to learn and memorize odor-reward associations.

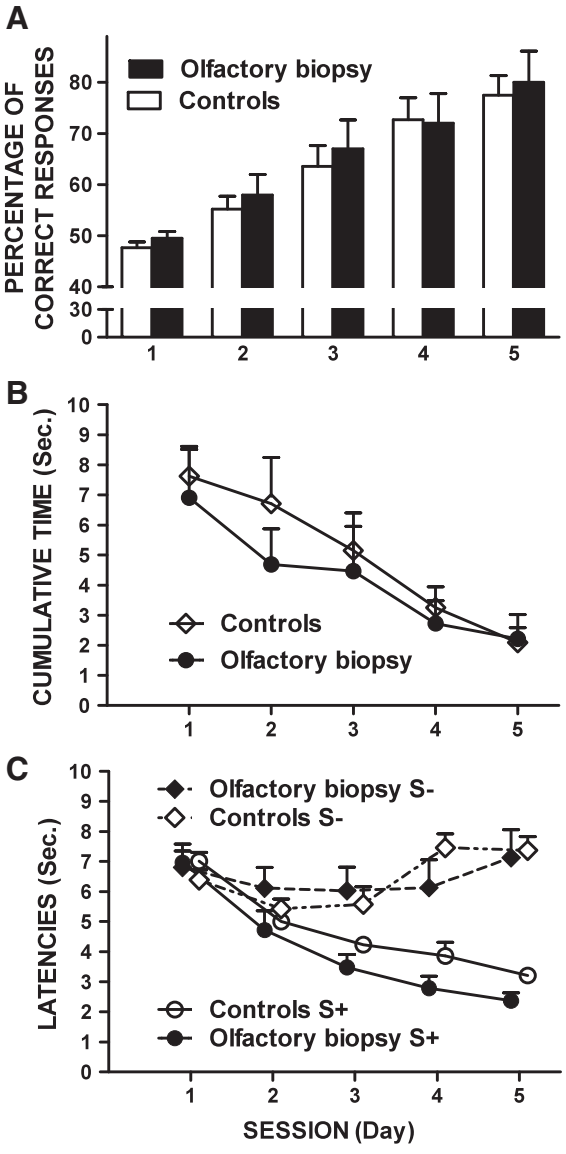

Figure 3 Effect of the biopsy on the sense of smell and on associative memory performance. Behavioral mean performance (±SEM) obtained across five sessions of 60 trials in "Controls" and excised rats ("olfactory biopsy") (n = 8 for both groups). (A) Mean percentage of correct responses for each session. (B) Mean cumulative time (in seconds). (C) Mean latencies (in seconds), S+ being the latencies for the positive odor and S− the latencies for the negative odor. No significant statistical difference was observed between the two groups throughout the successive five sessions, whatever the analyzed parameters.

Conclusions

This unique technique developed in rats allows in less than 15 min to biopsy olfactory mucosa, without significant impairment in odor discrimination. We show here that olfactory stem cells can be expanded in vitro. It should however be pointed out that the same technique can be used for cultivating other olfactory cell types of interest, especially the olfactory ensheathing cell. The latter can be easily amplified in culture, as demonstrated before (Bianco et al., 2004; Richter et al., 2008). Overall, the currently reported protocol will allow experiments based on autologous grafting of nasal olfactory cells, paving the way for clinical trials in humans.

Acknowledgments

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