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Immunohistological and electrophysiological characterization of Globose basal stem cells

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ABSTRACT

Objective(s): In the past few decades, variety of foetal, embryonic and adult stem and progenitor cells have been tried with conflicting outcome for cell therapy of central nervous system injury and diseases. Cellular characteristics and functional plasticity of Globose basal stem cells (GBCs) residing in the olfactory epithelium of rat olfactory mucosa have not been studied in the past by the neuroscientists due to unavailability of specific markers for GBCs. In the present research, we standardized some techniques to isolate GBCs from rat olfactory epithelium in pure form using a highly selective GBC-III antibody passaged through fluorescence activated cell sorter (FACS). We also characterized these cells immunohistologically using various pluripotent stem cell markers. This work also throws some light on ionic channels present on these stem cells which are responsible for their neuron induction potential.

Materials and methods: Globose basal stem cells were isolated from rat olfactory epithelium using GBC-III antibody and were characterized as multipotent stem cells using various neural progenitor markers. Ionic channels on GBCs were studied with voltage clamping.

Results: GBCs could be isolated in pure (99% purity) form and were found to be stained positive for all neural progenitor cell markers. Voltage gated Na^+ channels were completely absent, which proves the unexcitable nature of GBCs. Leaky K^+ channels were found to be present on the GBC which was of no significance.

Conclusion: This research work can be helpful in understanding the nature of these stem cells and utilising them in future as potent candidates for neuro-regenerative therapies.

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Introduction

Neurogenesis is a process during which new neurons are generated from a precursor cell. This process requires proliferation, differentiation and migration of neurogenic stem cells into neurons. For many decades, it was believed that in mammals, neurogenesis occurs only during embryonic life, but a recent work has made it evident that neuronal regeneration occurs even after birth in specific tissues like the hippocampus, dentate gyrus, subventricular zone and olfactory epithelium (1). Damage to the olfactory periphery destroys the population of olfactory sensory neurons and, in the case of direct epithelial lesion, also eliminates other constituents of the epithelium. In marked contrast to other parts of the nervous system, there is substantial anatomical and functional recovery of the olfactory epithelium and its projection into the CNS even in the face of overwhelming injury (2). Years, neuroscience researchers have searched for an ideal cell with the properties of absolute totipotency and regenerability in vitro and *in vivo*. Science has come very close to the quest, but still many unanswered questions remain. Recent studies indicate that transplantation of pure stem cell population is insufficient for attaining the maximum positive effect of neural regeneration. More complete recovery of the structure, sensory and motor functions of injured spinal cord can be attained using a complex of cells, including, apart from olfactory ensheathing cells (OEC), fibroblasts, astrocytes, schwann cells, and olfactory epithelial (OE) multipotent stem and progenitor cells. Hence, the problem of the efficiency and the possibility of using various OE cells and tissues for transplantation therapy in cerebral and spinal injuries doubtlessly requires further experimental studies. Nevertheless, consistent efforts with increasing optimistic outcome are being made to identify a candidate (neuron- progenitor cell) for a reliable therapeutic intervention of central

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nervous system (CNS) injury. In this venture, recent years witnessed an increasing bang on the olfactory epithelium for suitable multipotent stem cells. Multiple numbers of pluripotent stem cell candidates have been tried in the past for neuro-regenerative therapies with unconvincing results (3). Studies involving exposure of olfactory epithelium (and causing its lesion) to an irritant like methyl bromide (MeBr) have shown that the olfactory epithelium is restored its normal status that is indistinguishable from unlesioned epithelium within 6–8 weeks after damage with no relation to the severity of the initial damage which could be up to 90% of the epithelium being destroyed. The first sign of regeneration of olfactory neurons appears on the 4th day after MeBr exposure, the first mature neurons emerge during the 2nd week after which there is an accelerated production of neurons which falls to normal around the 6th week after the lesion (2). The reconstitution of the epithelium is sufficiently robust and precise so that the spatial distribution and the numbers of odorant receptors (OR) are restored to normal (4). Transplantation of adult animal and human OE cells have been used in the past for experimental and clinical correction of spinal injuries (5). The adult olfactory epithelium (OE) is a unique (for its capacity to renew olfactory receptor neurons throughout adult life) and a complex tissue containing heterogeneous population of epithelial cells. Apart from the support cells and neruoreceptor cells, this complex is said to retain a kind of progenitor cells that are competent to make neurons (neural stem cells) and non- neural support cells like olfactory ensheathing cells, oligodendrocytes and schwann cells (6). These support cells, play a role in the regeneration and myelination process of normal and of injured CNS. Recent studies identified stem like characteristics in a group of cells called Globose Basal Cells (GBCs) residing in the basal compartment of the olfactory epithelium. The GBCs are the small, round, morphologically non-descript and cyto-keratin negative cells that sit between the horizontal basal cells (HBCs) below and the immature olfactory receptor neurons above, that proliferate at a high rate in the normal OE, are limited to the OE, and are poorly characterized at the level of their molecular phenotype. Further, a second population of cells that are called the HBCs with stem - cell like properties has been described to reside among the GBCs. Therefore, it appears that the olfactory epithelium contains at least two populations of cells with possible neuropotency (7). Some studies have shown that there is an increase in the population of GBCs and not the HBCs during the regenerative process of the olfactory epithelium (8). GBCs function as broadly multipotent progenitors capable of giving rise to neurons and all of the cell types of the epithelium, and hence may be totipotent stem cells of the epithelium. Marker studies using antibodies that are selective for GBCs in normal epithelium, during the acute phase in the recovery after MeBr exposure, suggest that the GBCs are differentiating into non-neuronal cell types (9). Intranasal infusions of GBCs derived from retroviruses, give rise to neurons and multiple types of non-neuronal cells, while other stem cell progenitors give rise only to non-neuronal cells (10). GBCs that are separated from the normal epithelium by cell cytometry using a GBC-selective primary antibody and are pure and free from other cells and contaminants, engraft easily into the MeBr-lesioned epithelium after infusion into the nasal cavity and give rise to most of the neural and non-neural cells present in the OE (11). Embryonic stem cells (3), haematopoetic stem cells and other mesenchymal stem cells have enormous ability to differentiate into different types of cells in the body; but they are still the possibilities of immune rejection, adverse effects of immunosuppressive therapy and many ethical issues which exclude them as an ideal candidate for clinical practice (12). Moreover, these different varieties of stem cells do not reside in a niche similar to the neural stem cells and hence cannot proliferate easily in the central nervous system. On the other hand, olfactory epithelial cells share the same environmental niche as the central nervous system and also have the same developmental hierarchy as the neural cells. Olfactory mucosa is readily accessible, easily biopsied for autologous cell transplantation and regenerates completely without any loss of function. These properties make the olfactory epithelial stem cells the ideal candidate for use in clinical research and therapy. Since the basal compartment contains two morphologically distinct cell types, horizontal basal cells (HBCs) and globose basal cells (GBCs), there has been arguments regarding the identification of the neural stem cell population which cannot be answered merely based on their location and mitotic activity and studies focusing on search for unique stem cell progenitor markers have to be undertaken (8, 13, 14). Researchers in the recent years have proposed the most likely lineage of the cells in the olfactory epithelium by using specific markers, determination of various transcription factor expressions and incorporation of analytical studies using ^{3}H-thymidine and bromodeoxyuridine which suggest the proliferative activity of dividing cells (15, 16). It is now clear that immature ORNs are generated from one of the basal cell types and they in turn give rise to mature ORNs, but the identity of these cells has to be further characterised. Only in limited experiments, it has been shown that GBCs are the cell population to multiply and regenerate vigorously after epithelial damage. There is still an uncertainty of the lineage relationship between the two basal cell populations.

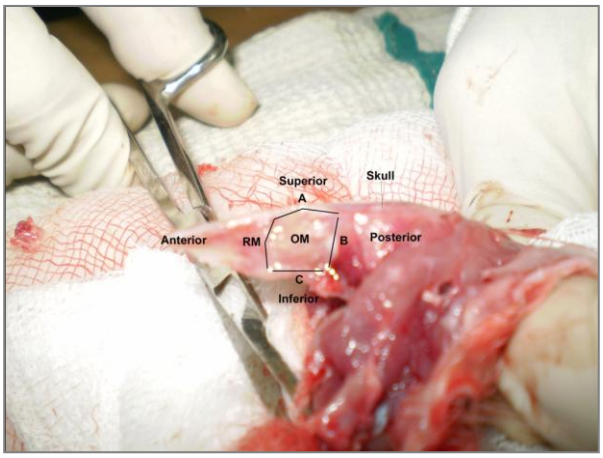

Figure 1. Nasal septum with the olfactory mucosa is exposed. 3 lines marked. A-arc of perpendicular plate, B-cribriform plate, C-ceiling of oral cavity. OM-olfactory mucosa (yellowish), RM-respiratory mucosa (pinkish)

In this research, we study different neural progenitor markers and ionic channels that are present on the GBCs and hence characterize these cells as definitive mutipotent stem cells residing in the basal compartment of rat olfactory epithelium capable of transforming into neurons and non-neuronal elements.

Materials and Methods

Separating olfactory epithelial cells

Six albino Wistar rats were purchased from the animal facility of Christian Medical College Vellore after obtaining the clearance from the animal ethics committee. Albino Wistar rats are the most commonly used laboratory animals for genetic and cell line studies as many aspects of their behaviour and physiology are similar to humans and this similarity can be easily observed in them when compared to other animals (17). This strain of rats was used in this study since their olfactory mucosa is easily accessible and their olfactory epithelium is histologically similar to that of the human. All animals were weighed prior to the experimental (94 -120 g, with an average of 105 g). The olfactory mucosa of the rats was obtained by surgical excision under complete, universal sterile conditions. The albino Wistar rats were anesthetized by intraperitoneal administration of 90 mg/kg body weight of ketamine and 10 mg/kg body weight of xylazine. The nasal septum was completely excised along three lines: the arc of the perpendicular plate, the cribriform plate and the ceiling of the oral cavity to obtain the olfactory mucosa (Figure 1). The nasal septum along with the olfactory mucosa was placed in frozen Dulbeco's modified Eagle's medium (DMEM) containing antibiotics (penicillin, streptomycin and amphotericin). The nasal septum along with the olfactory mucosa was then treated with 0.5 ml of Dispase II and was incubated at 37˚C for 45 min. The olfactory epithelium was separated from the lamina propria on both sides of the nasal septum using a micro spatula under a Leica EZ2HD dissecting microscope. The olfactory mucosa was mechanically dissociated with a scalpel blade and treated with 0.05% trypsin and an enzymatic cocktail containing collagenase –1 mg/ml, hyaluronidase –1.5 mg/ml and trypsin inhibitor – 0.1 mg/ml to separate the cells. The OE cells were transferred to epithelial culture medium which was modified form of the previously described medium (18-20). The culture medium was composed of DMEM / F12 (1: 1) –47.5 ml, 2% fetal bovine serum (Invitrogen, Gibco, India) – 1.0 ml, N2 supplement (Invitrogen, Gibco, India) –0.5 ml, epidermal growth factor (25 ng/ml – Invitrogen, Gibco, India) –12.5 µl and L-glutamine (Invitrogen, Gibco, India) –0.5 ml.

Isolating pure GBCs

Ten µl of GBC III antibody was added to the epithelial cell pellet under dark conditions and was left in ice for 45 min. GBC-III is a mouse monoclonal IgM antibody which recognizes a 40 kDa surface antigen which is a laminin receptor surface protein. It is highly specific as a marker for GBCs, unlike the earlier antibodies used like GBC-I which were nonspecific markers for GBCs and showed positive reaction even with HBCs, Sus and duct cells (21). GBC-III in powdered form was reconstituted into the recommended volume of 250 µl. It was used at a concentration of 1:100 for immunostaining. Ten µl of R-phycoerythrin-conjugated Affinipure $(Fab')_2$ fragment goat anti-mouse IgM (secondary antibody) was added to the cell pellet and left under dark conditions in ice for 10 min. Fluorescence assisted cell sorting (FACS) was used to separate the GBCs from other epithelial cells. Finally, 99% pure GBCs were obtained using FACS and GBC-III antibody and were plated in the epithelial medium.

Electrophysiological characterization

Ionic channels of pure GBCs were characterized using the voltage clamping method. GBC cell suspension in the epithelial medium was washed 3 times with Hanks balanced salt solution. Cells were trypsinised using 100 µl of 0.1% trypsin/EDTA and incubated at 37°C for 5 min. Epithelial medium was added immediately after incubation to neutralize the enzymes and the cell suspension was centrifuged at 1000 rpm for 8 min. The supernatant was discarded and 250 µl of epithelial media was added. A Poly D-Lysine plated cover slip was placed in a 35 mm petridish. The cell suspension was then plated on this cover slip and incubated for 2 hr. Micropipettes were prepared using F-500 fine point microforge to have the tip diameter of 1 micron. Micropipette tips were coated with Sylgard to reduce capacitance currents generated by the micropipette. The cover slip coated with the cells was washed with ECF (extra cellular fluid) and was placed in a 35 mm petridish filled with ECF.

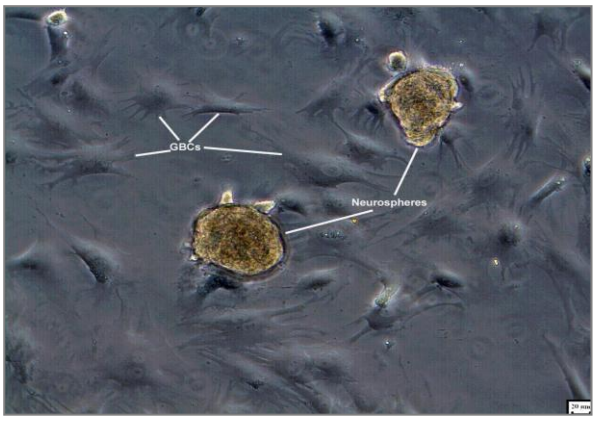

Figure 2. Phase contrast image showing 2nd generation of neurospheres on the 14th day in epithelial medium

Composition of the neuron solution ECF was NaCl – 135 mM, KCl – 4 mM, NaH_2PO_4 – 1 mM, $CaCl_2$ –1.2 mM, $MgCl_2$ – 0.5 mM, HEPES – 10 mM and Glucose – 10 mM. pH was titrated to 7.4 with 1 M NaOH. All the micropipettes were filled with ICF (intra cellular fluid) before being attached to the pipette electrode. Composition of the neuron solution ICF was KCl – 140 mM, EGTA – 1 mM, $MgCl_2$ – 1 mM, HEPES – 10 mM and glucose - 10 mM**.** pH was set at 7.2 using 1 M KOH. The petridish was placed on the stage of a phase contrast microscope and the bath electrode was introduced into the ECF. The micropipette tip was gently approximated to the cell membrane of the GBC and a negative suction was given to form a seal. The average seal resistance for the GBCs was between 1.2 M ohm and 2 M ohm. Multiple sweeps were taken to calculate the current flow and analysed using the Clampex Scope Episodic software.

Immunohistochemical characterization

Immunostaining of pure GBCs was done with multiple numbers of neural progenitor stem cell markers to characterize GBCs as neural progenitor cells. Primary antibodies used for immunostaining were anti-NCAM, anti-Nestin, anti-Sox-1, anti-Sox-2, anti-CD29, anti-CD54 and anti-CD73. Secondary antibodies used were goat anti-mouse IgG 1 and goat ant-rabbit IgG (PerCP-conjugated AffiniPure $F(ab)_2$ fragment). Seven 35 mm petridishes were filled with phosphate buffered saline (PBS). Cover slips (coated with poly D-lysine) plated with the GBCs were washed in PBS 3 times. Cells were fixed by treatment with 4% paraformaldehyde for 15 min at room temperature. Cover slips were again washed 3 times with PBS. 7 glass slides were prepared and kept moist for mounting the cover slips. Cover slips were placed on the slides and blocked using 2% goat serum (blocking agent) mixed with 0.1 % Triton X (permeating agent) for 2 hr at room temperature. After blocking, the slides were washed 3 times with PBS and treated with the primary antibodies and incubated overnight at 4˚C. Slides were then treated with the respective secondary antibodies and incubated at 37°C for 1 hr. The slides were then examined under immunofluorescence microscope. Dilutions used for different primary antibodies were as follows; anti NCAM- 1:50, anti Nestin- 1:20, anti Sox 1- 1:100, anti Sox 2- 1:5, anti CD29- 1:50, anti CD54- 1:50 and anti CD73- 1:50.

Results

The basal cells in the olfactory epithelium are believed to be the easily accessible autologous stem cells in the adults. However selection and isolation of the specific basal cells which are progenitors for neural cells are still being studied. The present research work helped us to isolate and decipher numerous new facts about the globose basal stem cells in the olfactory epithelium of rats.

Culture media and neurosphere formation

Culture medium used for growing and obtaining maximum confluency of the olfactory epithelial cells was standardized. The epithelial medium was composed of chemicals, enzymes and factors essential for epithelial cell proliferation and neurosphere formation. It further enhanced the capability of the cells to propagate and form neurospheres. The desirable viable cell density for obtaining optimum growth of epithelial cells on the media was calculated to be 10×10^4. Multiple neurospheres were formed by the end of 2nd week (Figure 2).

GBCs in pure form

As it is shown in Figure 3, 99% pure GBCs were obtained using FACS and GBC-III antibody. FACS Diva 6.0 software was used to analyse the data using graphs. The overall GBC density was calculated to be 52.1%. Total number of GBCs sorted was 257,000. The highest number of GBCs was found in the area with the total cell density of 4×10^3 (Figures 4 and 5).

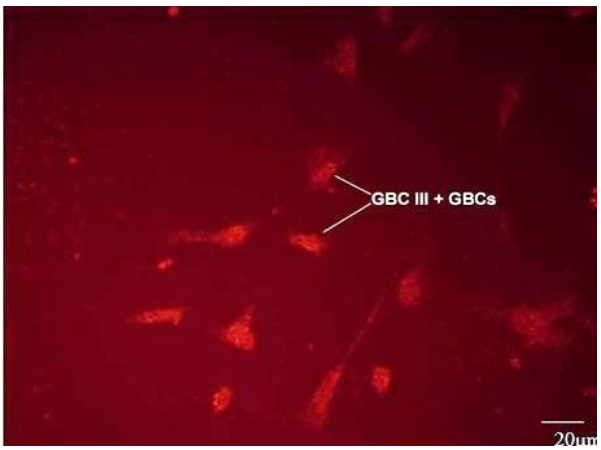

Figure 3. Immunofluorescent microscopic picture showing GBC stained with GBC-III antibody

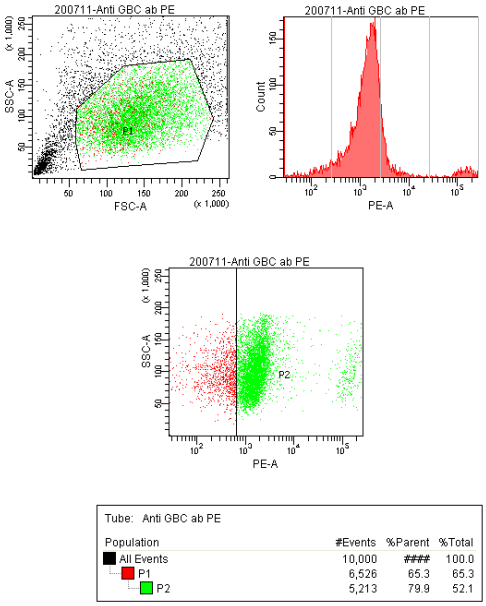

Figure 4. FACS Diva 6.0 graph showing P1 and P2 areas. Green- GBC-III positive cells; Red- GBC-III negative cells

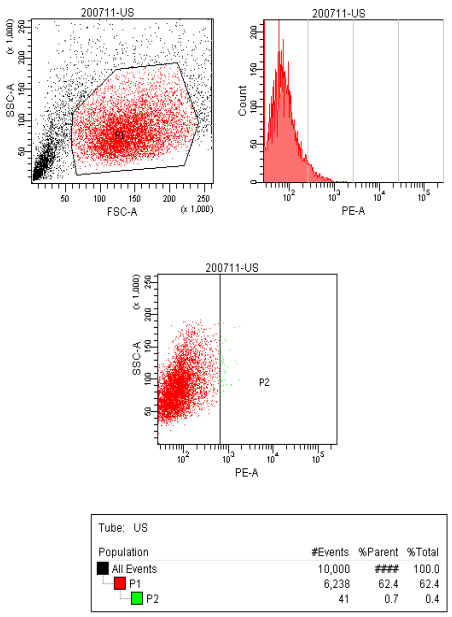

Figure 5. FACS Diva 6.0 of unstained cells used as negative control. Green- GBC-III positive cells; Red- GBC-III negative cells

Immunostaining

GBCs sorted by FACS were immunohistochemically characterised using various neural progenitor cell markers. Immunostaining of GBCs was positive with anti-NCAM (Figure 6, Va), anti-nestin (Figure 6, Vb) and anti-Sox 2 (Figure 6, Vc), hence confirming that these cells contain intracellular proteins like NCAM, nestin and Sox 2 and are neural progenitor cells. GBCs staining was also positive for CD-29 (Figure 6, Vd), CD-54 (Figure 6, Ve) and CD-73 (Figure 6, Vf) which are cell surface markers present on a variety of cells including neural progenitor cells. Marker study with Sox-1 was negative probably because of reduced number of GBC in that respective well.

Patch clamping

Electrophysiological characterisation of the isolated GBCs was done using patch clamping (Figure 7). Whole cell voltage-clamp recording from GBC was obtained using 60% series resistance compensation, following correction. Series resistance used was 0.6 M ohms. Thirty sweeps were taken with a holding

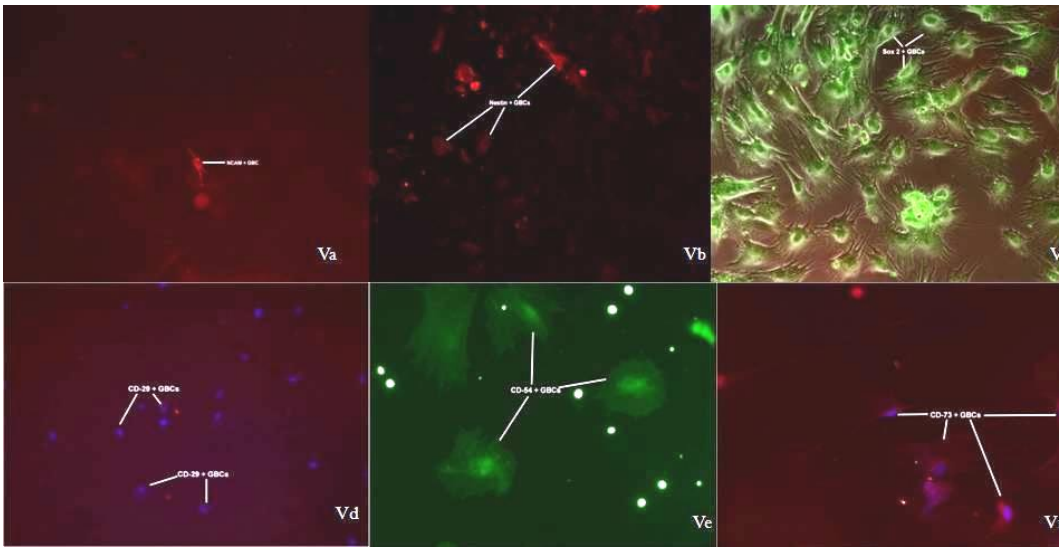

Figure 6. Va: Immunofluorescent image of a GBC stained with anti-NCAM antibody (10X)
Vb: Immunofluorescent image showing nestin positive GBCs (10X)
Vc: Immunofluorescent image showing Sox-2 positive GBCs (20X)
Vd: Immunofluorescent image of CD-29 positive GBCs (10X). Nuclei is stained with DAPI
Ve: Immunofluorescent image of CD-54 positive GBCs (40X)
Vf: Immunofluorescent image of CD-73 positive GBCs (20X). Nuclei stained with DAPI

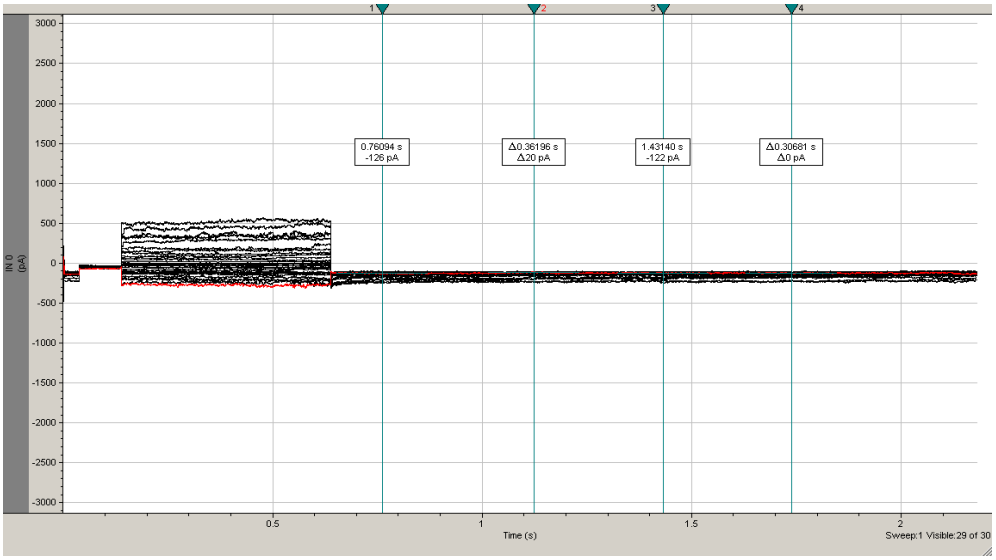

Figure 7. Whole cell voltage clamp recording from GBC. 29 0f 30 sweeps are seen

voltage of -80 mv. Also, +1 mv increments were applied starting from -40 mv to -10 mv to check for opening of any voltage gated Na^+ (sodium) channels. Voltage gated Na^+ channels were completely absent, hence proving the unexcitable nature of GBCs (Figure 8). Then, +10mv increments were applied starting from -150 mv to +150 mv to check for opening of any voltage gated K^+ (potassium) channels. A smooth I-V curve was not obtained and only leaky K^+ channels were found to be present on the GBC which was of no significance (Figure 9).

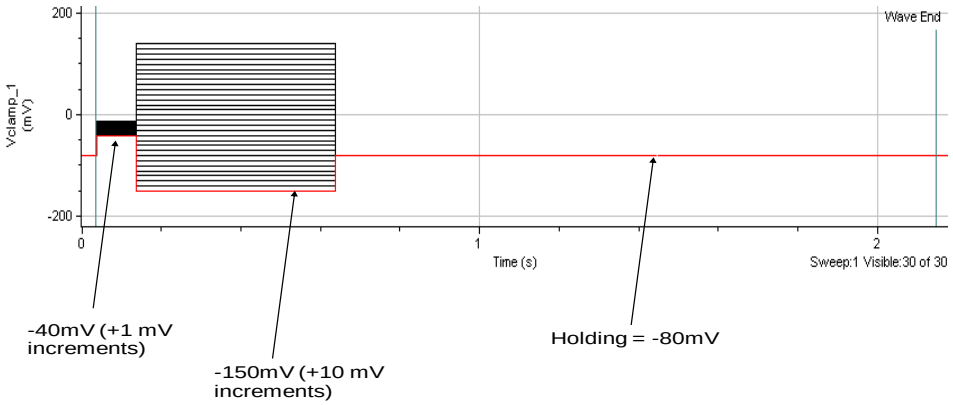

Figure 8. Left arrow- Na channels (closed); Middle arrow- leaky K channels; Right arrow- holding voltage

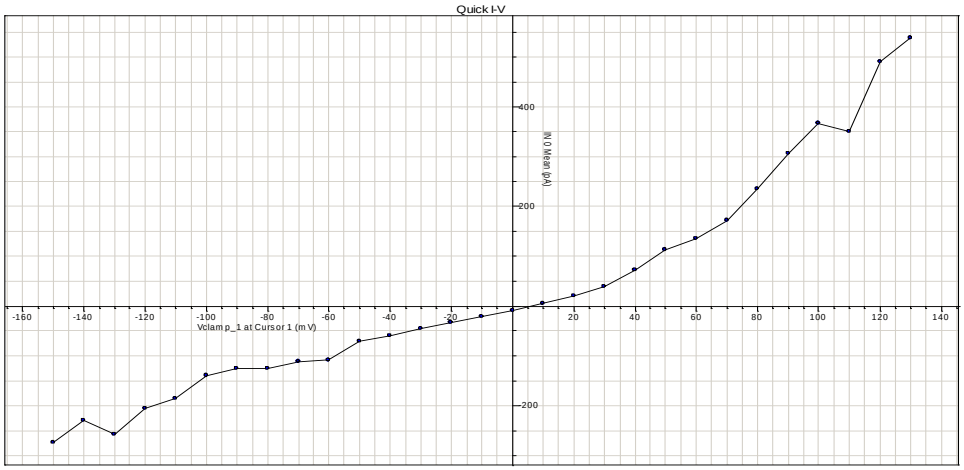

Figure 9. I-V curve of potassium current showing leaky channels. No smooth parabolic curve seen

Discussion

Neurogenesis begins in the early embryonic period (4th week) and continues 10 to 12 years after birth (22). The nervous system development is completed only during the adolescent period when myelination of the neurons of major tracts is completed (23). Early researchers believed the nervous system to be fixed and incapable of proliferation and regeneration based on the their opinions that neurons and non-neuronal cells in the nervous system could not divide actively via mitosis and they lacked the potential to transform from simple to more complex forms of neuronal morphologies. Another reason for such belief was inability of researchers in the past to demonstrate progenitor stem cells residing in the nervous system niche by specific markers. Cells in the nervous system could be demonstrated only by negative staining which was very non-specific (18, 22, 23). The earliest demonstration of neurogenesis in adults was done by Joseph Altman in 1962 in the cerebral cortex (24) followed by demonstration of neurogenesis in the dentate gyrus of the hippocampus in 1963 (25). Later, it was evident that neurogenesis also occurred in the subgranular zone and subventricular zone of the lateral ventricles (26). Now, researchers also believe that neurogenesis continues in the cerebellum during adulthood (27). The most recent development in this field of research is the demonstration of neurogenesis in the olfactory system including the olfactory bulbs and more importantly in the olfactory mucosa.

This research work studies one such population of stem cells residing in the olfactory epithelium which has the niche required to get accepted in the CNS environment and is derived from the same germ line as the neuronal cells. This gives a hint that these might be the ideal neural progenitor cells which can be easily harvested and used for autologous transplantations.

Olfactory epithelial- mesenchymal stem cells have been isolated in the past from the olfactory mucosa using enzymatic dissociation with collagenase IA (28) in an impure form. Isolated cultures of GBCs and horizontal basal cells have been grown in earlier experiments by immunoadhesion (29).This research work standardizes chemical methods to isolate these stem cells from the olfactory epithelium in a pure form. Epithelial cells were harvested on the culture media after calculating the feasible cell densities for optimal growth and neurosphere formation. Densities higher than this resulted in overcrowding of cells and loss of cells due to unwanted cell death. Densities lower than this did not provide us with adequate number of cells and hindered neurosphere generation. The self renewal potential of olfactory stem cells was evident from the formation of multiple generations of neurospheres. During 2 weeks, many of the neurospheres started to proliferate. Hence, an effective method of generating neurospheres was established. EGF and N-2 supplement seem to play a significant role in providing an optimum external niche for formation of multiple generations of neurospheres from epithelial stem cells. EGF is a potential stem cell mitogen allowing cell proliferation through activating signalling pathway and inducing the production of beta fibroblast growth factor (βFGF) from the progenitor cells. It was also established that neurosphere generation depended on the initial plating density. Olfactory mucosa in cultures has been shown to produce certain soluble factors which help with proliferation, regeneration and neurosphere formation (30).

GBCs were isolated in the pure form using FACS and cell densities were calculated. The area P1 was chosen with relatively higher percentage of GBC-III stained cells and a graph was plotted showing the GBC density to be 65.3% of the total number of cells. P2, a part of P1, with the highest cell density was chosen and a similar graph was plotted which showed the GBC density to be 79.9% of the total (Figure 4). Unstained cell suspension was also run through FACS as a negative control. An insignificant number of 41 positive cells (accounting for 0.4% of the total) were sorted from the negative control proving the efficacy of the procedure (Figure 5). GBCs have been isolated in the past from other progenitor cells of the olfactory epithelium using FACS by tagging them with less sensitive and specific antibodies like GBC-I (9) and GBC-II (29). The complexities of the phenotypical stages of transformation of the GBCs into olfactory neurons upon neuroablation of the olfactory bulb have been explained using enhanced green fluorescent protein tagged with markers like SOX2 and Neurog1 (31).

Further research on GBCs and other potential neural progenitor cells present in the olfactory mucosa will help us to understand their biology and effectiveness in neurogenesis. It will also help to apply these results to medical use for therapeutic treatment of different diseases. Few areas on which special attention can be drawn in future are:

- Isolation of GBCs from the OE using magnetic assisted cell sorting (MACS). MACS has never been used earlier to separate GBCs. Hence, its usage will help us to compare its efficacy with FACS and a better purity of cells can be obtained (32).
- Electrophysiological studies to find out more about different ion channels present on the GBCs will help us to have a better understanding of the functional properties of these cells and their drug interactions.
- Animal studies using GBCs to repair the spinal cord injuries and other nervous system disorders should be the ultimate aim to propagate and

effectively utilize the present knowledge on these cells in human trials.

Conclusion

This research work has been able to throw some light on the prevailing ambiguity of the olfactory stem cells and has derived some definite conclusions about the nature and behaviour of these cells. Non-excitable nature of GBCs has been documented in the present study. Positive expression of all the neural progenitor cell markers has proved substantially about the neuro-transformation capability of the GBCs. Now, after undertaking this study, we can conclude with concrete explanations that GBCs are the colony of neural stem cells residing in the basal compartment of the olfactory epithelium responsible for the ongoing neurogenesis in the OE throughout adult life.

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