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## Regeneration of New Neurons is Preserved in Aged Vomeronasal Epithelia

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### Abstract

During normal and diseased aging, it is thought the capacity for tissue regeneration and repair in neuronal tissues diminishes. In the peripheral olfactory system, stem cell reservoirs permit regeneration of olfactory and vomeronasal sensory neurons, a unique capacity among neurons. Following injury a large number of new neurons can be regenerated in a young animal. However, it is unknown whether this capacity for renewal exists in aged proliferative populations. Here we report that neuronal replacement associated proliferation continues in the vomeronasal organ of aged (18-24 months of age) mice. In addition, the potential for the aged stem cell to yield a mature neuron persisted at the same rate as that observed in young animals. Furthermore, the robust regenerative capacity to respond to both acute and sustained injury following olfactory bulbectomy remains intact even in very old animals. Hence, the neuronal epithelium lining the vomeronasal organ is unique in that it contains stem cells capable of generating functional neurons throughout life and in the aged animal in particular. This persistent regenerative capacity provides optimism for neuronal replacement therapies in the aged nervous system.

### Keywords

stem cell; regeneration; olfactory; renewal; reconstitution; vomeronasal

### Introduction

Stem cell technology and therapy presents particular difficulties in the nervous system since virtually all stem cell activity ceases relatively early in the brain's development. There are few exceptions, including the subgranular and subventricular proliferative zones that supply new neurons to the hippocampus and the olfactory bulb, respectively (Whitman and Greer, 2009). However, the cells in these regions of the brain are difficult to purify and manipulate; because of their apparently programmed identity they may have diminished future therapeutic value. In addition to these regions, the epithelium that lines the vomeronasal organ (VNO) contains cells capable of lifelong proliferation.

During normal and diseased aging, it is thought the capacity for regeneration and repair in neuronal tissues diminishes (Mimeault and Batra, 2009) due to impairments in gene expression and the function of the stem cell population (Bailey et al., 2004; Ahlenius et al., 2009; Wagner et al., 2009). Olfactory epithelia are unique in that stem cell reservoirs permit regeneration of olfactory and vomeronasal sensory neurons and that the generated cells are

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excitatory projection neurons (Graziadei and Monti Graziadei, 1983; Halpern and Martinez-Marcos, 2003). Until recently, studies of the effects of aging and neuronal regeneration have generally been limited to animals no older than 2-3 months of age with little attention given to aged adult animal models. It is likely that stem cell therapies will require isolation from an aged system, as the onset of most neurodegenerative diseases is during later life stages. It is therefore desirable to understand how the proliferative capacity of stem cells is altered with aging.

The epithelium of the VNO is an especially favorable system to investigate these issues. The VNO is a bilaterally symmetrical tubular structure located at the base of the septum containing a pseudostratified columnar epithelium composed of basal cells, intermediate neuronal precursors, mature sensory neurons, and sustentacular cells. The sensory neurons are CNS Golgi type I neurons with a long axon and glutamatergic synapses in the olfactory bulb (Firestein, 2001). The olfactory and vomeronasal epithelia are highly similar in the cell types represented (Mendoza, 1993). Basal cells are adult stem cells that retain the capacity to regenerate new neurons throughout life, and are the primary cell type labeled by thymidine analogs (Schwartz Levey et al., 1991; Huard and Schwob, 1995; Halpern and Martinez-Marcos, 2003). The basal cell population gives rise to MASH1<sup>+</sup> progenitors and subsequently Neurogenin-1<sup>+</sup> immediate neuronal precursors. In the VNO, precursors migrate both horizontally and vertically to become mature sensory neurons, which express both olfactory marker protein (OMP) and neural cell adhesion molecule (NCAM) (Calof et al., 2002; Halpern and Martinez-Marcos, 2003). Two types of stem cells have been identified in the VNO. Cells found in the marginal zone are largely responsible for growth, while cells found in the central zone are associated with neuronal replacement (Fig. 1; Weiler et al., 1999; Martinez-Marcos et al., 2000a,b; Weiler, 2005; De La Rosa-Prieto et al., 2009). We therefore sought to determine the capacity for neuronal growth and regeneration in the two stem cell populations in an aged environment. In particular we asked whether and to what extent neuronal stem cells found in the aging vomeronasal system retain their capacity to regenerate in the face of diminishing tissue growth.

## Materials and Methods

### Animal Care & Sources

C57BL/6 mice, aged 1 to 24 months, were obtained from the NIA Aged Rodent Colony. All experiments were performed in compliance with the Columbia University Institutional Animal Care and Use Committee.

### Immunohistochemistry (IHC)

Mice were anesthetized by intraperitoneal injection with a mixture of ketamine/xylazine (0.05-0.15 ml 18 mg/ml, and 2 mg/ml, respectively) and perfused transcardially with heparinized (2-4 unit/ml) phosphate buffer saline (PBS) (pH 7.4) followed by 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M phosphate buffer (PB) (pH 7.4). Vomeronasal epithelia were dissected, decalcified, cryoprotected (30% sucrose) and frozen in OCT. Cryosections (12  $\mu$ m) were incubated in blocking solution containing 0.5% Triton X-100 and 5% normal donkey serum in 0.1 M PB for 1 hour at room temperature. Primary antibody incubation was performed overnight at 4°C against olfactory marker protein (OMP; goat polyclonal; Wako, Richmond, VA; 1:1500), 5-bromo-2'-deoxyuridine (BrdU; mouse monoclonal; GE Healthcare Biosciences, Piscataway, NJ; 1:30), and active caspase-3 (rabbit polyclonal; Cell Signaling, Danvers, MA; 1:200). Sections were then incubated with Alexa Fluor 488 and 594 conjugated secondary antibodies (Molecular Probes, Eugene, OR; 1:750) for 2 hours at room temperature. TOTO-3 (Invitrogen, Carlsbad, CA; 1:10,000) was added in a wash step after secondary incubation for

visualization of nuclei. Sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA) to prevent photobleaching. Optical sections (1  $\mu\text{m}$ ) were taken through the depth of the section with an Olympus FluoView 600 (Melville, NY) confocal microscope and analyzed with ImageJ software. Figures were assembled with Adobe Photoshop and Illustrator software.

### Olfactory Bulbectomy

Olfactory bulbectomy (OBX) was performed according to standard methods. Briefly, mice were anesthetized by intraperitoneal injection with a mixture of ketamine/xylazine (0.05-0.15 ml 18 mg/ml, and 2 mg/ml, respectively). A small hole was cut in the frontal bone over the right olfactory bulb with a dental drill, and the bulb was removed by aspiration. The cavity was filled with Gelfoam (Pfizer Pharmacia & Upjohn Co., Kalamazoo, MI) to control bleeding, and the skin over the wound was sutured closed with Vetbond (3M, St. Paul, MN).

### Proliferation Assay

Proliferation was assessed by BrdU (Sigma, St. Louis, MO) injection and was detected with an antibody recognizing BrdU (described above). For acute labeling experiments, 100 mg/kg BrdU was injected two hours prior to sacrifice. For maturation studies (see below), two injections two hours apart of 50 mg/kg BrdU were given; the animals were then sacrificed 30 days following injection. For olfactory bulbectomy studies, 100 mg/kg was injected two hours prior to sacrifice on the fifth or thirtieth day after OBX was performed.

### Maturation Assay

Cryosections were prepared as described above (see *Immunohistochemistry*). In all animals, at least 15 coronal sections throughout the VNO were examined to yield a minimum of twenty cells examined per animal. Optical sections (1  $\mu\text{m}$ ) were taken with a 40 $\times$  objective through the depth of the section with an Olympus FluoView 600 confocal microscope and analyzed with ImageJ software. Complete overlap in all visual planes with a nuclear marker TOTO-3 was required in order to verify that examination was of a single BrdU-labeled cell. Z-projections through the middle of BrdU/TOTO-3 positive nuclei were then made to verify that OMP labeling surrounded the entire nucleus. Cells that exhibited partial labeling with OMP or those in which the plane of cryosection bisected the nucleus were not included in the analysis.

### Stereology and Quantification

Thin cryosections (12  $\mu\text{m}$ ) were prepared as described above (see *Immunohistochemistry*). In all animals, every fifth section (60  $\mu\text{m}$  increments) of vomeronasal epithelia were processed for BrdU and OMP immunohistochemistry, counted immediately with a Leica DMR microscope (Leica Microsystems, Bannockburn, IL) to minimize effects of photobleaching, and photographed with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI). Adjacent sections were processed for active caspase-3 and OMP immunohistochemistry. The area of OMP immunoreactivity was used to define the reference space and was measured with ImageJ (NIH) software to allow for normalization of counts. Counts were performed with the trained experimenter blind to condition in an unbiased, random manner with systematic sampling. Total number of BrdU-positive cells was not calculated but rather the number of BrdU-positive cells per unit area, as BrdU labeling observed at the dosages utilized herein is a rare event.

## Reconstruction

The rostral and caudal extent of the VNO was determined in each animal. Cell counts were made for each position (see *Stereology and Quantification*), and each position was normalized to the total length of the VNO, such that the rostral end of the epithelium corresponded to 0% and the caudal end to 100%. For each 5% increment of VNO length, cell counts were pooled and averaged for each animal, as more than one section was counted for the range of each 10% increment. For each age group, cell counts were again averaged across all animals in that age group according to 10% increments in position. Finally, as absolute cell numbers labeled by BrdU decline with age in the marginal zone, for each position the percent contribution to the total BrdU-positive cell count along the length of the VNO was determined. Data were graphically represented as heatmaps with MathWorks MatLab software.

## Statistical Analysis

All statistical analyses were performed with GraphPad Prism software (GraphPad Software, La Jolla, CA). For analysis of basal proliferation levels, all data were analyzed for statistical significance by one-way ANOVA and Student Newman-Keuls (SNK) pairwise multiple comparison between age groups. Where raw data did not pass Bartlett's test for equal variances, data were log transformed and subsequently analyzed by ANOVA as stated above (Fig. 2B; Table 1, all zones and marginal zone). For reconstruction studies (described in Fig. 4), percent contribution at a given position was analyzed for statistical significance with a two-way ANOVA for the effects of age and position, followed by Bonferroni posthoc tests for the effect of position in all age groups. For maturation studies, data were expressed as a ratio defined by the number of cells that were both OMP-positive and BrdU-positive divided by the number of BrdU-positive cells, regardless of OMP status. As maturation ratio data were not normally distributed, they were arcsin transformed and analyzed with a one-way ANOVA and SNK pairwise multiple comparison between age groups. For olfactory bulbectomy studies, lesion and non-lesion control measurements were performed in the same tissue section to control for effects of position. As variances between lesion and non-lesion control were unequal in all age groups, raw data were normalized to lesion values and transformed with an arcsin transformation. Normalized data were analyzed with two-way repeated measures ANOVA for the effects of age and lesion, followed by Bonferroni posthoc tests between lesion and non-lesion control for each age group.

## Results

In order to assess how proliferation is affected by age, we utilized 5-bromo-2'-deoxyuridine (BrdU) as a marker of cell proliferation. BrdU is a thymidine analogue that is incorporated into the DNA of proliferating cells during the S-phase of the cell cycle, and provides a reliable, quantifiable immunoreactive signal. We chose BrdU instead of other proliferative markers such as Ki-67 or proliferating cell nuclear antigen (PCNA) because they are not specific to the phase of the cell cycle, exhibit non-specific persistence beyond proliferation, or are subject to variability with fixation (Ohta and Ichimura, 2000; Muskhelishvili et al., 2003; Taupin, 2007). We injected 100 mg/kg BrdU two hours prior to sacrifice in mice in nine age groups, ranging from 1 to 24 months of age. The C57BL/6 mouse lifespan ranges up to an extreme of approximately 30 months of age (Konen et al., 1973). A non-saturating dose of BrdU was chosen to avoid experimental confounds of toxicity (Cameron and McKay, 2001; Taupin, 2007). In coronal sections, the VNO was subdivided into three zones: the marginal zone (M), the intermediate zone (I), and the central zone (Ce) (Fig. 1A, right panel; 1B, merge; Giacobini et al., 2000). Utilizing stereological quantification of cells incorporating BrdU, we observed that the majority of immunoreactivity was in the basal cell layer. In addition, a minor level of proliferation occurred in the sustentacular (or supporting)

cell region, clearly recognizable due to the elongated nuclei of sustentacular cells relative to the compact circular nuclei of the neurons found along the periphery of the epithelium. Proliferation in the sustentacular layer remained constant throughout the life of the animal (data not shown;  $P > 0.05$ ). In all age groups, a large proportion of BrdU incorporation was in the marginal zone (Fig. 1B; 2A,C). When counts from the basal cell layer in all zones were considered together, we observed that proliferation decreases sharply following 2 months of age, and plateaus from 3 to 24 months of age (Table 1; one-way ANOVA,  $P < 0.0001$ ). When data were analyzed separately by zone, proliferation decreased sharply with age in the marginal (Fig. 2A,C; Table 1; one-way ANOVA,  $P < 0.0001$ ) and intermediate (Table 1; one-way ANOVA,  $P < 0.0002$ ) zones. However, in the central zone, proliferation remained constant throughout life (Fig. 2B,D; Table 1; one-way ANOVA,  $P > 0.05$ ). In tissue sections adjacent to those analyzed for BrdU immunoreactivity, cell death was also quantified. In both the marginal (Fig. 3B) and central (Fig. 3C) zones, the number of cells immunoreactive for active caspase-3 (aCasp3; Fig. 3A) was not different across all age groups ( $P > 0.05$ ). Interestingly, all aCasp3 immunoreactivity observed was localized to the mature neuronal layer and not observed in the basal cell compartment.

We next calculated the ratio between proliferation (BrdU-positive cells) and death (active caspase-3-positive cells) in both the marginal and central zones for each animal in each age group. In young animals (1-2 months), the ratio was highest in marginal zone, and this value was significantly different from that in the central zones. In adult and aged animals (6-24 months), the ratios in the two zones were not different (Fig. 3D). These data and the observed decline in growth-associated proliferation are consistent with the fact that horizontal migration from the marginal zones decreases by 2 months of age (Halpern and Martinez-Marcos, 2003) and is similar to the timing of the growth-associated decline observed in the olfactory epithelium (Hinds et al., 1984). The consistent level of cell death observed in all age groups further indicates the initial elevated amount of BrdU-incorporating cells in the marginal zone is due to growth during a restricted early postnatal period. Other factors, such as the microenvironment that supports these progenitor cells, may cause the growth-associated decline in neurogenesis seen with age (Morrison and Spradling, 2008). Therefore, beyond the postnatal growth period, the level of constant proliferation and cell death observed in all age groups in the central zone likely results from vertical migration of new replacement neurons.

We next asked whether the distribution of BrdU incorporation throughout the rostral-caudal length of the VNO exhibited age-dependent changes. To do this, we reconstructed the pattern of labeling in the VNO following an acute 100 mg/kg BrdU injection from mice aged from 1 to 24 months. Across all age groups and all zones of the epithelium, the majority of BrdU incorporation was at the most rostral and caudal extents of the VNO (Fig. 4). Hence, the number of cells incorporating BrdU is highest at the extremities of the VNO, regardless of zone, and this pattern does not change with age ( $P > 0.05$ , two-way ANOVA for the effects of age and position).

As progenitor cells undergo proliferation, they generate more daughter cells than will mature into functional sensory neurons (Martinez-Marcos et al., 2005). It is not clear whether age affects the potential for newly generated cells to become functional neurons. To further probe this question, we injected mice, ranging in age from 1 to 24 months of age, with two doses of 50 mg/kg BrdU two hours apart on day zero. Mice were then sacrificed thirty days following BrdU injection. Single cells (as verified by the nuclear marker, TOTO-3) incorporating BrdU were subsequently examined with confocal microscopy for colocalization of OMP, a marker of neuronal maturity (Fig. 5B). No significant differences were observed between age groups ( $P > 0.05$ , one-way ANOVA), with approximately with one-third of the BrdU+ cells having become mature sensory neurons (Fig. 5A).

Since the proportion of mature sensory neurons does not decline with age, we next asked whether the regenerative capacity of this neural stem cell was retained in aged animals after injury. The regenerative capacity of both the olfactory epithelium and the vomeronasal epithelium of young animals has been studied extensively with several paradigms, including target ablation (unilateral olfactory bulbectomy), sensory deprivation (unilateral naris occlusion), axotomy and chemical ablation of the epithelium (for review, see Halpern and Martinez-Marcos, 2003). Lesion of the axons extending from the olfactory epithelium and VNO to the olfactory bulb by bulbectomy (OBX) results in a wave of apoptotic cell death that selectively targets all neurons in the epithelium within 5 days of the lesion. However, the robust regenerative capacity of the basal cells repopulates the epithelia with more than 8-10 million new neurons within 3-4 weeks (Costanzo and Graziadei, 1983; Schwartz Levey et al., 1991; Caggiano et al., 1994; Kastner et al., 2000; Yoshida-Matsuoka et al., 2000; Matsuoka et al., 2001, 2002; Carter et al., 2004).

Since previous lesion experiments were conducted in young animals, we asked whether this remarkable regenerative capacity in response to injury is lost with age. We first performed unilateral OBX in mice aged 2, 6, and 24 months. Five days after surgery (OBX5), mice were injected with 100 mg/kg BrdU two hours prior to sacrifice and their vomeronasal epithelia were examined for BrdU incorporation. In all age groups, we observed that proliferation in the basal cell compartment increased following lesion throughout all areas of the VNO (Fig. 6C, D) over that of the non-lesioned control (Fig. 6A, B). Following stereological quantification, we observed that proliferation significantly increased in all zones in all age groups after OBX over the non-lesioned control VNO (Fig. 7A-C;  $P < 0.01$  for the effect of surgery,  $P > 0.05$  for the effect of age, two-way repeated measures ANOVA for the effects of age and surgery). These data demonstrate that while the normal pace of proliferation slows over age, the capacity for robust proliferation remains latent and unchanged when challenged.

The olfactory bulbectomy lesion model requires the removal of the olfactory bulb and thus the target for the re-growing neurons is absent. In younger animals this sustained lesion results in the continuous turnover of sensory neurons as the newly generated cells fail to find a target and die. We were therefore interested in these continuing effects in the aged epithelium. To test this we again performed unilateral OBX in mice aged 1, 2, 6, 12, and 18 months. We then waited for 30 days post surgery (OBX30) before injecting mice with 100 mg/kg BrdU prior to sacrifice. In both young (2 month) and aged (18 month) mice, proliferation was increased throughout the lesioned VNO (Fig. 6G, H) over that of non-lesion control (Fig. 6E, F). When data were analyzed by zone, a significant increase in proliferation following lesion was observed in all zones of the VNO and in all age groups (Fig. 7D-F;  $P < 0.0001$  for the effect of surgery;  $P > 0.05$  for the effect of age, two-way repeated measures ANOVA for the effects of age and surgery). These data show that the capacity for long-term neuronal regeneration from adult stem cells is undiminished even in very old animals.

## Discussion

Regeneration and proliferation of neurons in adults and particularly aged organisms could have significant clinical implications for restoring brain function due to injury or pathology. Although the young brain has already limited regenerative capacity, it has been thought that in the aged brain this capacity is further diminished. We have shown here that, among a population of excitatory projection neurons, this is not the case.

The phenomenon of neurogenesis has been most commonly investigated with thymidine analogues such as BrdU to label proliferative cell populations. The loss of BrdU

immunoreactivity following high doses of irradiation (Taupin, 2007; Valley et al., 2009) suggests that BrdU incorporation does not typically reflect DNA repair. In addition, a study examining the specificity of BrdU incorporation following three well-characterized models of injury (including irradiation, olfactory bulbectomy, and kainic acid-induced seizure) provides additional evidence that BrdU is not significantly incorporated during DNA repair. BrdU incorporation occurred principally in cells undergoing DNA synthesis. In particular, in the olfactory epithelium following olfactory bulbectomy in young adult animals, dying cells were positive for TUNEL (Tdt-mediated dUTP-biotin nick end labeling), a cell death marker, but rarely incorporated BrdU (Bauer and Patterson, 2005). These data suggest that post-mitotic dying neurons do not re-enter the cell cycle following lesion and that BrdU administration is an appropriate experimental approach to monitor proliferation in the olfactory system.

A decline in neurogenesis with normal aging has been observed in the rare populations of adult neurogenic cells in the nervous system. In the central nervous system, the age-related decline in neurogenesis has been demonstrated in the subgranular zone (SGZ) that supplies new neurons to the dentate gyrus of the hippocampus (Rao et al., 2006) and the subventricular zone (SVZ) that supplies new neurons to the olfactory bulb (Ahlenius et al., 2009). The observed decline is due to a loss of progenitor populations in the SGZ (Olariu et al., 2007) but neurons generated from these remaining progenitors exhibit normal spine density in mice up to 10 months of age (Morgenstern et al., 2008). While maturation of progenitors derived from the SGZ was retarded, the migration, survival, and neuronal fate choice was normal in the aged rat (Rao et al., 2005). A similar result was observed in the SVZ, where the numbers (Luo et al., 2006) and potential of neuronal stem cells were diminished in aged mice (Maslov et al., 2004).

In the olfactory epithelium, several studies have demonstrated that neurogenesis continues into adulthood (Farbman, 1990; Mackay-Sim and Kittel, 1991; Fung et al., 1997). In general, while these studies do not examine neurogenesis in aged animals, a decline in the rate of neurogenesis has been observed with increasing postnatal age. For example, proliferation was examined up to 3 months of age in mice (Mackay-Sim and Kittel, 1991), and 11 months of age in rat (Weiler and Farbman, 1997, 1998). In addition, age affects the expression of regulators of the cell cycle in the olfactory epithelium (8.5 month old mice; Legrier et al., 2001). Similar to our findings in the mouse VNO, proliferation in the guinea pig olfactory epithelium was decreased by 4 months of age and was maintained at a constant level from 4-24 months. Interestingly, proliferation levels dropped after 24 months of age (Nakamura et al., 1998). However, this report only examined the guinea pig up to 36 months of age and the lifespan of this rodent ranges up to 72 months of age. Additionally, it is difficult to separate the difference between growth and cell replacement in the olfactory epithelium.

In the accessory olfactory system, including the VNO, there is a lack of data regarding the effects of aging on its regenerative capacity. The presence of proliferative cells in the normal snake (Holtzman, 1998), mouse (Martinez-Marcos et al., 2005) and lesioned mouse (Wakabayashi and Ichikawa, 2007) VNO is established, but the rodent studies utilize young (2-5 month old) adult animals and therefore do not query age as a specific variable. A report in rat VNO presents evidence of declining proliferation with age, but its oldest group was 24 months of age in a rodent with a typical lifespan of 36 months, and it did not assess the response to injury (Weiler et al., 1999).

The response to injury of aged neuronal tissue is of intense interest for both basic research and clinical intervention. The brain is particularly sensitive to damage at advanced ages following injury such as ischemia, and it is not understood why this is the case, nor how to



alleviate this sensitivity (Popa-Wagner et al., 2009). One possible solution is that the regenerative potential of neuronal stem cells may be exploited to repair damage inflicted by injury. However, to date, variable results have been observed following injury in the neurogenic regions of the nervous system. Stroke resulted in an increase in new neurons in the striatum, but not in the subgranular zone (Darsalia et al., 2005). In the SVZ, the intrinsic differentiation potential of neuronal stem cells in young adult rats was not altered following stroke; however, the response of aged adults was not examined (Liu et al., 2009). A postmortem analysis of aged human SVZ indicated that neuronal progenitors increase following ischemia, but it is difficult to assess cell dynamics or neuronal maturation in postmortem brain tissue (Macas et al., 2006). Our finding that an easily accessible neuronal stem cell population is able to robustly respond to a severe injury paradigm is in contrast to recent work in the hippocampus that has demonstrated that aged neuronal stem cells in the SGZ do not retain their capacity for neurogenesis following deafferentation by kainic acid (Shetty et al., 2010). However, we cannot draw conclusions about whether neurons in aged animals born in response to injury are able to extend their axons to the accessory olfactory bulb, as the lesion method requires removal of the target of these axons. However, newly generated neurons in very old animals do reach maturity as shown by expression of the marker OMP.

Several lesion models have been utilized to probe the proliferative capacity of olfactory and vomeronasal epithelia. In the olfactory epithelium, 6 month old mice were able to recover function following zinc sulfate lesion, which disrupts the structure of the epithelium, indicating that the neural stem cells were able to successfully reconstitute the tissue at this young adult stage (Ducray et al., 2002). Here, we performed olfactory bulbectomy, a lesion that severs the axons of the vomeronasal and olfactory sensory neurons and induces programmed cell death specifically in the sensory neuron population (Schwartz Levey et al., 1991; Carr and Farbman, 1992). Within a day of lesion, macrophages are recruited to remove dying cell debris (Suzuki et al., 1995). Macrophages secrete leukemia inhibitory factor (LIF) that causes an increase in basal cell proliferation (Nan et al., 2001; Getchell et al., 2002). At the same time, loss of sensory neurons results in the decrease of growth and differentiation factor 11 (GDF11), a member of the TGF $\beta$  superfamily and secreted negative regulator of neurogenesis (Wu et al., 2003). GDF11 likely controls the replication probability of immediate neuronal precursors (Lander et al., 2009). These factors, in concert with other secreted factors and cell cycle proteins, induce proliferation and cell maturation to result in the rapid repopulation (within 10 days) of the epithelia with new sensory neurons (Kastner et al., 2000). To the best of our knowledge, the expression of GDF11 has not been examined in aged mice. However, it is likely that it is acting similarly in the aged epithelium; normally, neurogenesis is kept at a low level, but is permitted to increase when needed to mount an injury response.

It has been suggested that the exposure of sensory neurons to environmental damage may require their regular replacement (Gaskell, 1990; Dahl and Lewis, 1993; Upadhyay and Holbrook, 2004; Yankner et al., 2008). However, we find here that stem cells of the more protected vomeronasal epithelium undergo regular neuronal proliferation, maturation and cell death as is observed in the more exposed olfactory epithelium. Additionally, we and others have now noted the difference between growth and replacement of damaged neurons, and this replacement is a very slow process under normal conditions. Nonetheless the proliferative capacity in response to damage remains robust even with advanced age. Furthermore the response to injury can be sustained; in the absence of a target tissue, the subsequent death of maturing neurons appears to provide a signal to the stem cells that proliferation should continue at a high rate. Similarly, a recent descriptive report of nestin expression in human olfactory epithelium of aged individuals exhibiting hyposmia and/or anosmia suggests that aged humans may also retain the regenerative capacity described here

(Minovi et al. 2010). The search for the signal, or signals, involved in the reconstitution of the olfactory epithelia must now be expanded to include aged animals. Interestingly, p53, a gene best known for its role in tumor suppression, controls the regenerative capacity of neuronal precursors in the subventricular zone (Medrano et al., 2007). It is possible the mechanism governing proliferation of neuronal stem cells in the peripheral olfactory system may include p53 or its family members.

Although the remarkable proliferative capacity of the olfactory system has been known for some three decades, the regulatory mechanisms, and indeed the functional purpose, have remained obscure. The studies described here extend this robust and nearly unique phenomenon to a population of very old animals. The potential for neuronal replacement through an adult stem cell population is now also extended into an aging neuronal population.

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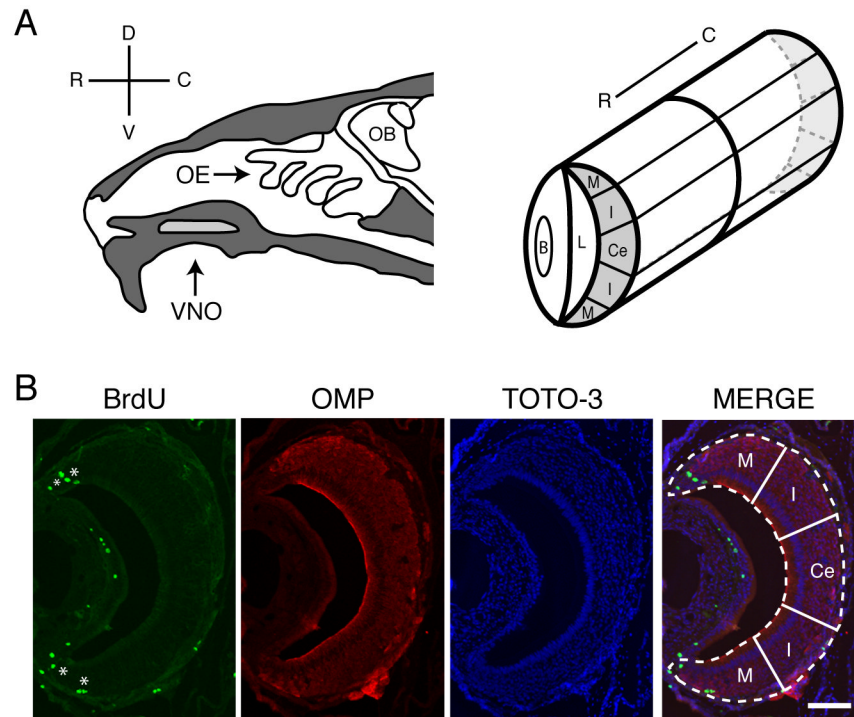
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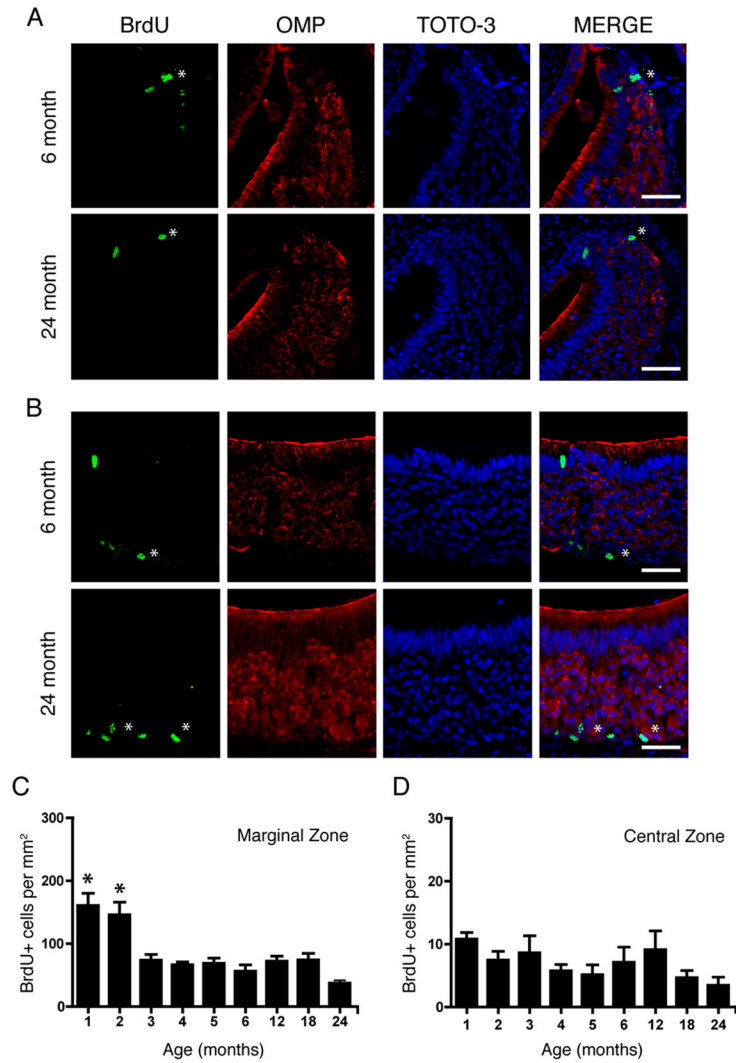
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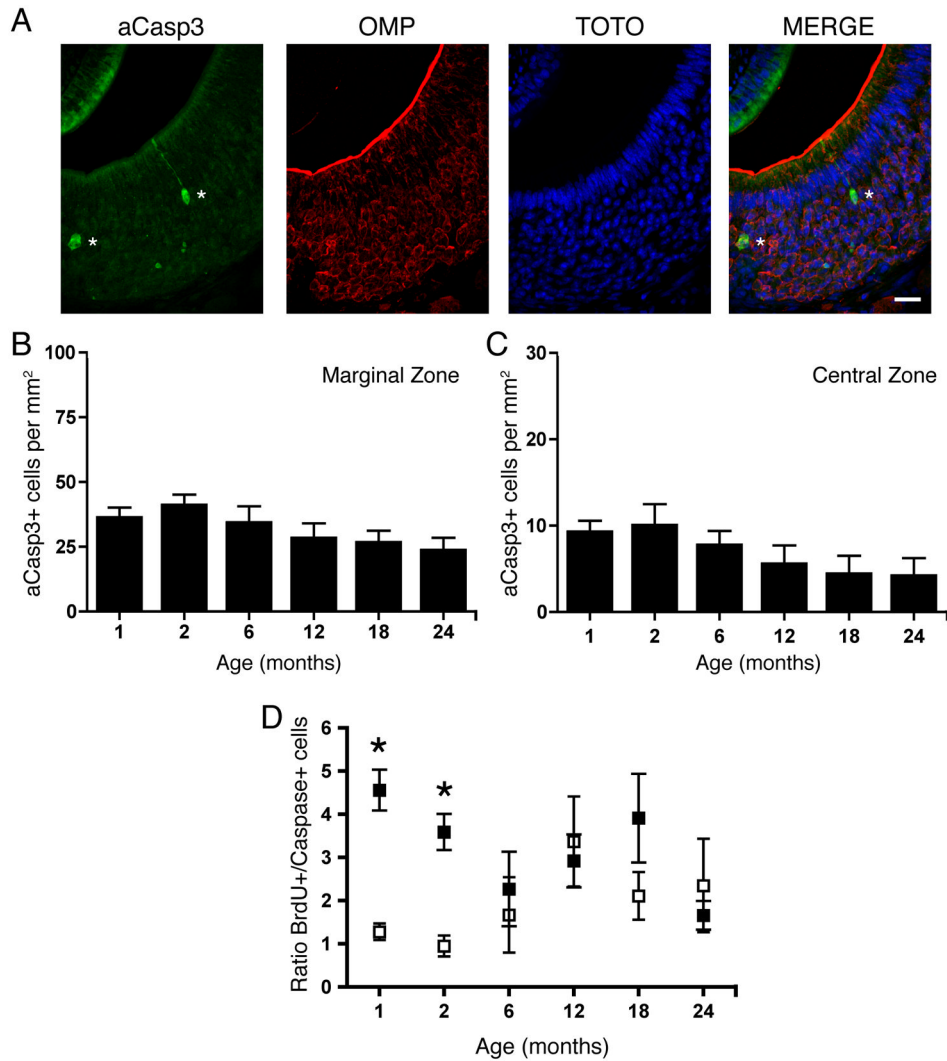
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**Figure 1.** Organization and zones of the vomeronasal organ. **A**, Schematics depicting the location of the VNO in the sagittal plane (left) and the coronal plane (right). The VNO is a bilaterally symmetrical tubular structure; shown here is one half of a coronal plane as it would appear through the depth of this structure. The marginal zones (M) are found at the extreme dorsal (D) and ventral (V) regions of the VNO. Adjacent to the marginal zones are the intermediate zones (I). In between the two intermediate zones is the central zone (Ce). Adapted from Firestein, 2001 and Mombaerts, 2004. Abbreviations: OB, olfactory bulb; OE, olfactory epithelium; VNO, vomeronasal organ; B, blood vessel; C, caudal; L, lumen; R, rostral. **B**, Incorporation of BrdU (green) in the VNO of a 2 month old mouse is shown. TOTO-3 is a nuclear marker (blue) and olfactory marker protein delineates the neuronal area of the VNO (OMP; red). Scale bar, 100  $\mu\text{m}$ .



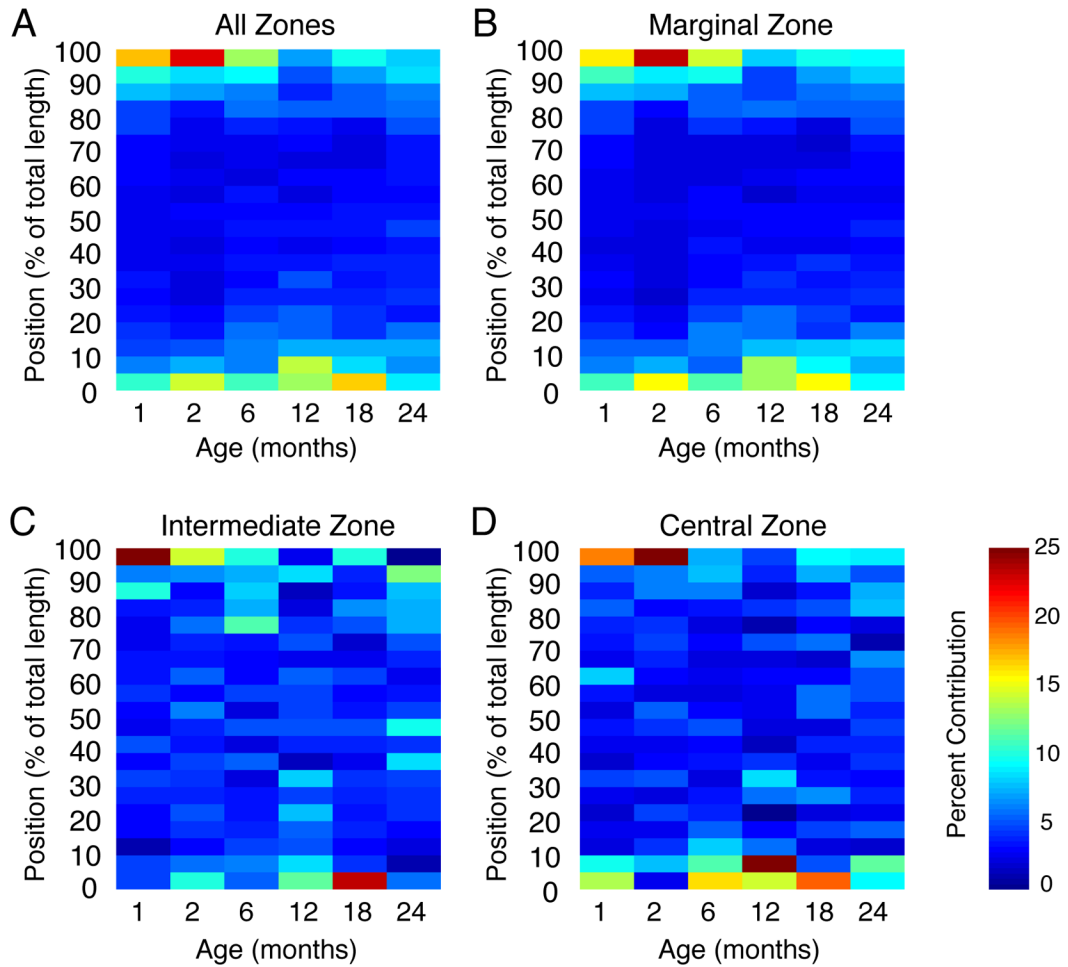
**Figure 2.** Proliferation decreases in the marginal zones but remains constant in the central zone of the VNO with age. **A**, Incorporation of BrdU (asterisks; green) in the marginal zone of the VNO of a 6 month old (upper panel) and a 24 month old (lower panel) mouse is shown in the merge of TOTO-3 (nuclear marker; blue) and olfactory marker protein (OMP; red). **B**, Same as in **A** but in the central zone of the VNO. **C**, Quantification of BrdU-positive cells per mm<sup>2</sup> in the marginal zone. **D**, Quantification of BrdU-positive cells per mm<sup>2</sup> in the central zone. All data were analyzed for statistical significance by one-way ANOVA and SNK pairwise multiple comparison between age groups. \*  $P < 0.0001$ ;  $n = 5-8$  animals per group. Scale bar, 50  $\mu\text{m}$ .



**Figure 3.**

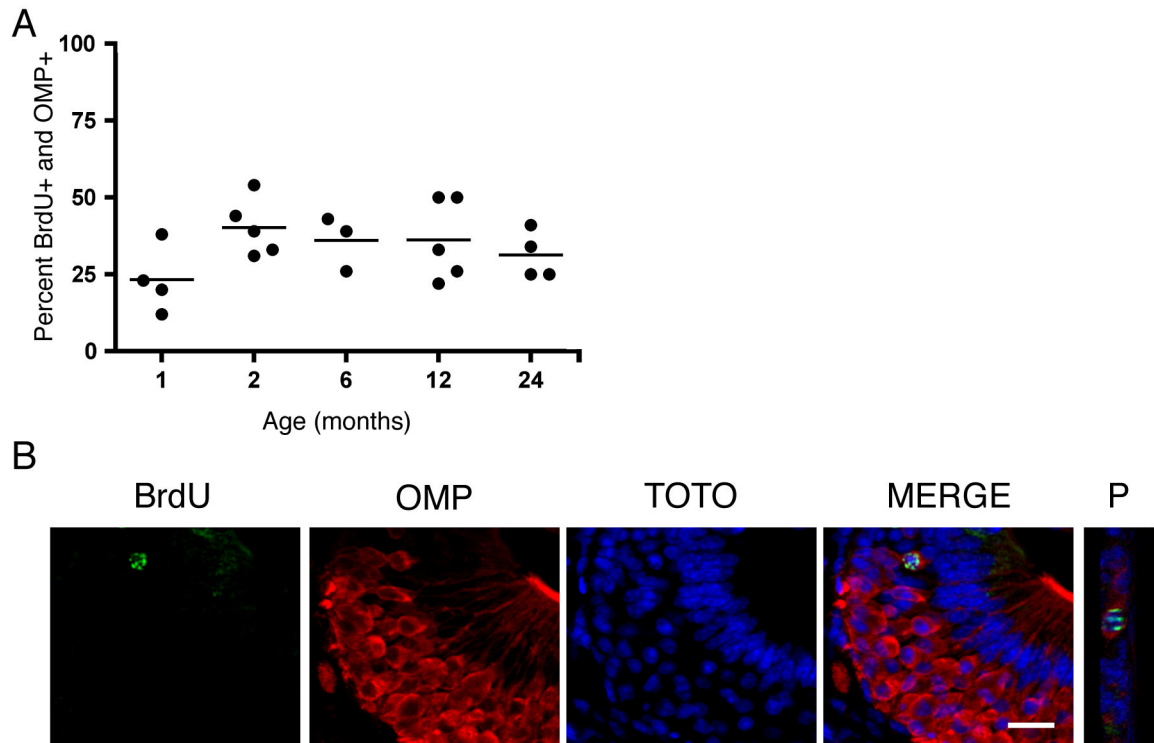
Cell death is constant in both the marginal and central zones of the VNO with age. **A**, Two cells expressing active caspase-3 (aCasp3; left panel; green), a marker of cell death, in the VNO of a 1 month old mouse are marked with asterisks. Also shown is olfactory marker protein (OMP; red), TOTO-3 (nuclear marker; blue), the merge of the three images (right panel). Scale bar, 20  $\mu$ m. Quantification of aCasp3-positive cells per mm<sup>2</sup> in the marginal (**B**) and the central (**C**) zones revealed no significant difference in the amount of cell death across all age groups. Data were analyzed for statistical significance by one-way ANOVA and SNK pairwise multiple comparison between age groups.  $P > 0.05$ ;  $n = 5-8$  animals per group. **D**, The ratio of proliferating cells to dying cells in the marginal zone (filled squares) is significantly different (denoted by asterisks) from that in the central zone (open squares) in young mice (1 month,  $P < 0.001$ ; 2 month,  $P < 0.005$ ) but not in aged mice (6-24 months,  $P > 0.05$ ). The ratio of BrdU-positive to active Caspase-3-positive cells was calculated for each animal in the marginal and central zones and analyzed for statistical significance by a paired Student's *t*-test.





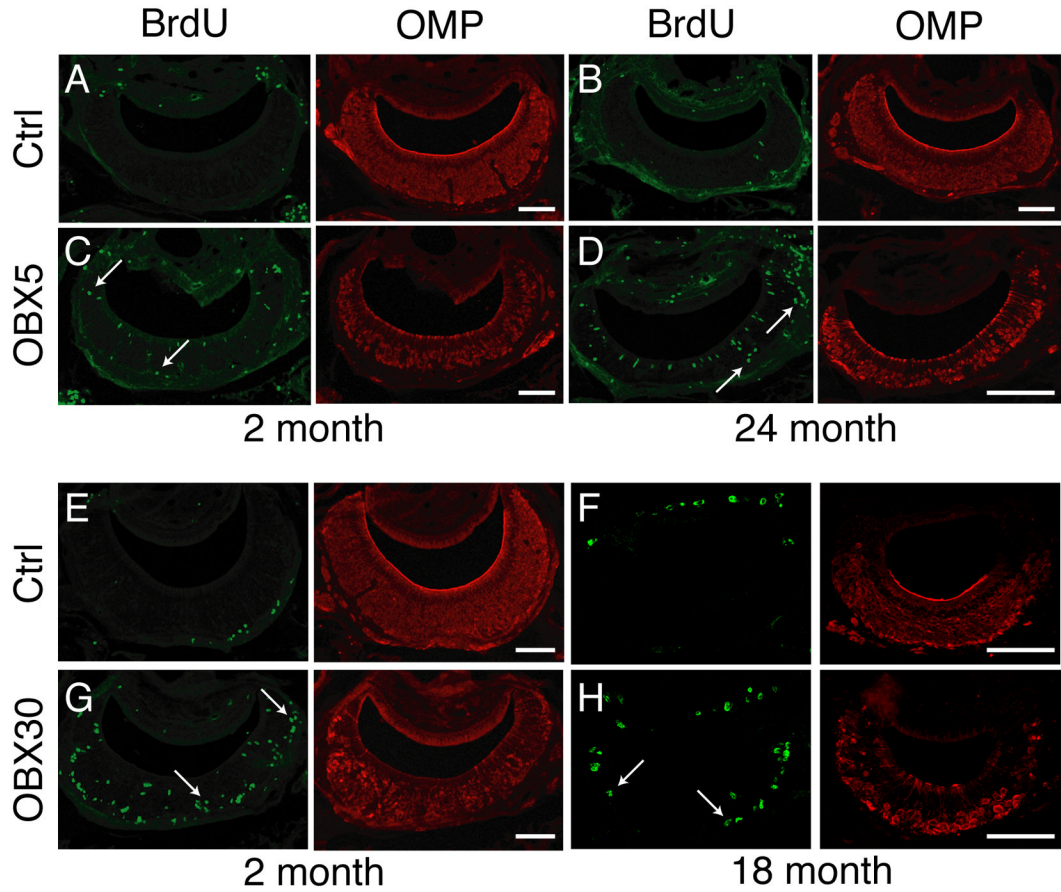
**Figure 4.**

Distribution of proliferating cells does not change with age. 100 mg/kg BrdU was injected 2 hr before sacrifice. BrdU counts were averaged for each 5% increment along the normalized length of the VNO for each animal in each age group ( $n = 5-7$  per group), with position 0% corresponding to the most rostral extent and position 100% corresponding to the most caudal extent of the VNO. Percent contribution of each position to the total number of BrdU-positive cells in each animal was calculated. The color represents percent contribution values as shown in the scale bar, with red corresponding to high percent contribution and blue corresponding to low percent contribution. Incorporation of BrdU is highest at the rostral (0%) and caudal (100%) extents of the VNO when data were analyzed either as all zones together (**A**) or separated by the marginal zone (**B**), the intermediate zone (**C**), or the central zone (**D**). No effect of age was observed in any of the zones. All normalized data were analyzed for statistical significance by two-way ANOVA for the effect of age (not significant) and position ( $P < 0.0001$  in **A**, **B**, **D**;  $P < 0.005$  in **C**).

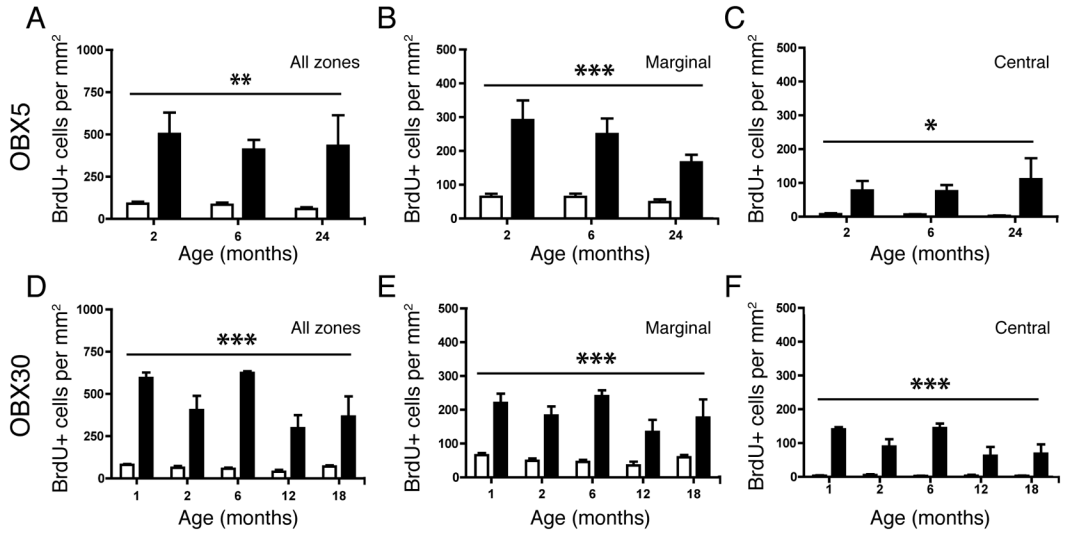


**Figure 5.**

Cells incorporating BrdU become mature sensory neurons at the same rate in both young and aged VNO. Mice were injected twice with 50 mg/kg BrdU two hours apart, sacrificed 30 days following injection, and cells incorporating BrdU were analyzed for the co-expression of olfactory marker protein (OMP), a marker of vomeronasal sensory neuron maturity. **A**, The percentage of cells incorporating BrdU that reach maturity (OMP-positive) is consistent across all age groups tested (1, 2, 6, 12, and 24 months of age; one-way ANOVA,  $n = 3-5$  animals per group,  $P > 0.05$ ). A horizontal line represents the mean for each group. **B**, Representative confocal images of a BrdU-positive cell (green) surrounded by OMP (red). Z-projections of all BrdU immunoreactive cells were made (P, projection) and examined for overlap with OMP and TOTO-3 (blue, nuclei) to verify that OMP immunoreactivity surrounded a single BrdU-positive nucleus. Scale bar, 20  $\mu\text{m}$ .



**Figure 6.** Acute and sustained unilateral olfactory bulbectomy (OBX) lesion induces increased proliferation in the VNO in young and aged mice. BrdU was administered 2 hr before sacrifice on the fifth day (OBX5; **A-D**) or the thirtieth day (OBX30; **E-H**) following OBX. **A**, BrdU (green) and OMP (red) in non-lesioned control 2 month old VNO. **B**, as in **A** but for control 24 month old VNO. **C**, BrdU (green) and OMP (red) in lesioned (OBX5) 2 month old VNO. **D**, as in **C** but for lesioned 24 month old VNO. **E**, BrdU (green) and OMP (red) in non-lesioned control 2 month old VNO. **F**, as in **E** but for control 18 month old VNO. **G**, BrdU (green) and OMP (red) in lesioned 2 month old VNO. **H**, as in **G** but for lesioned (OBX30) 18 month old VNO. Note the increase in BrdU incorporation throughout the all zones of the VNO (arrows) in all age groups. Scale bar, 100  $\mu$ m.



**Figure 7.**

Acute and sustained unilateral olfactory bulbectomy (OBX) lesion induces increased proliferation in all zones of the VNO in young and aged mice. BrdU was administered 2 hr before sacrifice on the fifth (OBX5; **A-C**) and thirtieth (OBX30; **D-F**) day following OBX. **A-C**, Quantification of BrdU incorporation reveals a significant increase in proliferation in the VNO following acute OBX (black bars) over that in the non-lesion control (white bars) in **A**, all zones (\*\*  $P < 0.003$ ), the **B**, marginal zone (\*\*  $P < 0.0003$ ) and **C**, central zone (\*  $P < 0.01$ ) regardless of age ( $n = 3$  per group). **D-F**, Quantification of BrdU incorporation reveals a significant increase in proliferation in the VNO following sustained OBX (black bars) over that in the non-lesion control (white bars) in **(D)** all zones (\*\*\*)  $P < 0.0001$ , **E**, marginal zone (\*\*\*)  $P < 0.0001$  and **F**, central zone (\*\*\*)  $P < 0.0001$  regardless of age ( $n = 3-7$  per group). All data were analyzed by two-way repeated measures ANOVA for the effects of surgery ( $P$  values noted above for each zone) and age (not significantly different).

**Table 1**  
**Proliferation across all zones of the VNO as it varies with age (BrdU-positive cells/mm<sup>2</sup>)**

| Age (months) | All Zones * § | Marginal Zone * § | Intermediate Zone † | Central Zone |
|--------------|---------------|-------------------|---------------------|--------------|
| 1 (n = 6)    | 195.9 ± 19.2  | 160.7 ± 19.3      | 24.4 ± 4.3          | 10.8 ± 1.1   |
| 2 (n = 7)    | 181.6 ± 24.9  | 145.9 ± 20.2      | 23.6 ± 4.5          | 7.5 ± 1.4    |
| 3 (n = 5)    | 95.8 ± 12.6   | 74.0 ± 9.0        | 13.2 ± 3.1          | 8.6 ± 2.7    |
| 4 (n = 5)    | 83.7 ± 4.2    | 66.6 ± 4.6        | 11.4 ± 1.5          | 5.8 ± 1.0    |
| 5 (n = 5)    | 85.4 ± 11.4   | 68.8 ± 8.3        | 11.4 ± 2.6          | 5.2 ± 1.6    |
| 6 (n = 7)    | 74.3 ± 13.4   | 56.6 ± 9.9        | 10.6 ± 2.3          | 7.1 ± 2.4    |
| 12 (n = 6)   | 91.7 ± 11.8   | 72.3 ± 8.2        | 10.3 ± 2.4          | 9.1 ± 3.0    |
| 18 (n = 8)   | 87.9 ± 11.3   | 74.3 ± 10.2       | 8.8 ± 1.3           | 4.7 ± 1.1    |
| 24 (n = 6)   | 48.0 ± 7.5    | 37.7 ± 4.7        | 6.8 ± 2.1           | 3.5 ± 1.3    |

Values are presented as mean ± standard error of the mean (S.E.M.). All data were analyzed for statistical significance by one-way ANOVA and SNK pairwise multiple comparison between age groups.

\* p 0.0001;

† p 0.0002.

§ Raw data did not pass Bartlett's test; data were log transformed and analyzed by ANOVA as stated above.