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Abstract

During mammalian embryonic development, GnRH neurones differentiate from the nasal placode and migrate through the nasal septum toward the forebrain. We previously showed that a category of glial cells, the olfactory ensheathing cells (OEC), forms the microenvironment of migrating GnRH neurones. Here, to caraterize the quantitative and qualitative importance of this glial, we investigated the spatio-temporal maturation of glial cells *in situ* and the role of maturing glia in GnRH neurones development *ex vivo*. More than 90% of migrating GnRH neurones were found to be associated with glial cells. There was no change in the cellular microenvironment of GnRH neurones in the regions crossed during embryonic development as glial cells formed the main microenvironment of these neurones (53.4%). However, the phenotype of OEC associated with GnRH neurones changed across regions. **The OEC progenitors immunoreactive to brain lipid binding protein** formed the microenvironment of migrating GnRH neurones from the vomeronasal organ to the telencephalon and were also present in the diencephalon. However, during GnRH neurone migration, maturation of OEC to [GFAP+] state **(glial fibrillary acid protein)** was only observed in the nasal septum. Inducing depletion of **OEC in maturation, using transgenic**

mice expressing herpes simplex virus thymidine kinase driven by the *Gfap* promoter, had no impact on neurogenesis or on triggering GnRH neurone migration in nasal explant culture. Nevertheless, depletion of [GFAP+] cells decreased GnRH neurite outgrowth by 57.4%. This study suggests that specific maturation of OEC in the **nasal septum** plays a role in morphological differentiation of GnRH neurones.

Introduction

Glial cells play a key role in neural development and function. They guide neuronal migration in early development and participate in neurite extension, synaptic formation and transmission. In the central nervous system (CNS) there are two major types of glial cells derived from the neural tube, astrocytes and oligendrocytes. In the peripheral nervous system (PNS), Schwann cells are the main class of glia derived from the neural crest. Olfactory ensheathing cells (OEC) are a unique class of glial cells restricted to the main and accessory olfactory system (MOS, AOS) (Su & He, 2010; Geller *et al.*, 2013). They also derive from the neural crest and possess characteristics and functions of astrocytes and Schwann cells (Su & He, 2010; Forni *et al.*, 2011). During development OEC contribute to olfactory neurogenesis and/or olfactory axonal growth according to their phenotype. Recent research indicates OEC may play an important role in the migration of GnRH neurones which control reproductive function (Forni & Wray, 2015). These neurones originate from the olfactory placode (OP) during embryonic development. They migrate within the nasal septum (NS) along axons and nerves of the AOS from E11.5 (days of embryonic development), and turn ventro-caudally towards the forebrain to reach their final location, in the hypothalamus around E16.5 in the mouse (Forni & Wray, 2015). In a previous study we showed that OEC accompanied migrating GnRH neurones, from their exit of the vomeronasal organ (VNO) to the nasal forebrain junction (NFJ), and expressed molecules involved in GnRH neurone migration (Geller *et al.*, 2013). Further work on *Sox10* Knockout mice embryos has shown that a reduction in the number of OEC progenitors (pOEC) which are BLBP+ (brain lipid binding protein) in the NS causes a default of GnRH neurone migration but does not impact vomeronasal/olfactory axon formation (Barraud *et al.*, 2013; Pingault *et al.*, 2013). In $p75^{NTR}$ Knockout mice deleting $p75^{NTR}$ expression in GnRH neurones and in OEC has been shown to

modulate GFAP+ OEC maturation, GnRH cell survival and neuronal migration (Raucci *et al.*, 2013). Overall, these data suggest that disruption of OEC differentiation/maturation could cause a default of GnRH neurone ontogenesis. Surprisingly, in the AOS little research has described either the spatio-temporal distribution of BLBP+ pOEC or the kinetics of OEC development. Maturation of OEC from the progenitor state to their mature state has been linked to an evolution of markers. Among these markers, BLBP has been identified as a hierarchical progenitor to OEC (Murdoch & Roskams, 2007), while GFAP (Glial Fibrillary Acid Protein) as a marker of mature OEC (Astic *et al.*, 1998). In the present study using GFAP-GFP mice we characterized the spatio-temporal maturation of the glial microenvironment of migrating GnRH neurones in the AOS and forebrain from the BLBP+ to the [GFAP+] state. In addition, we assessed the direct involvement of maturing OEC (mOEC) in GnRH neurone development using GFAP-HSV-Tk mice.

Materials and Methods

Animals

The Jackson Laboratory provided the FVB/N-Tg (GFAP-GFP) 14Mes/J transgenic mouse strain, with the stock number: 003257. The transgene, described in Zhuo et al. (1997), encodes for the GFP-S65T protein under the control of the human glial fibrillary acidic protein (GFAP) promoter (Zhuo *et al.*, 1997). Homozygous transgenic mouse GnRH-GFP C57BL6/j was kindly provided by D. Spergel. The transgene described in Spergel et al. (1999) encodes for the GFP protein under the control of A 3.47 kb *Gnrh* promoter fragment. GFAP-HSV-Tk transgenic mice were provided by Jackson Laboratory (strain: B6.Cg-Tg(Gfap-TK)7.1Mvs/J). Transgenic mice expressing herpes simplex virus thymidine kinase (*HSV-Tk*) driven by the *Gfap* promoter were generated by Bush et al (1998). Treatment with the antiviral agent ganciclovir (GCV) preferentially ablated transgene-expressing proliferative [GFAP+] glial cells. Animals were housed in the *Unité Experimentale de Physiologie Animale de l'Ofrasière* (INRA, Tours, France) maintained on 12L:12D cycles and fed *ad libitum*. The day of the vaginal plug detection was considered as E 0.5. All experimental procedures and animals used were approved by the Val de Loire Ethics Committee for Animal Experiments (agreement number: 2012-06-18).

Nasal explant cultures

Nasal explant cultures were performed as previously described (Geller *et al.*, 2013). Nasal pits of embryonic E11.5-stage GFAP-HSV-Tk mice were dissected out. Nasal explants were adhered onto coverslips (Marienfeld) using chicken plasma/bovine thrombin clot (sigma-Aldrich) which allows three-dimensional cellular migration. The explants were maintained in a defined serum-free medium (SFM) (Geller *et al.*, 2013) with or without 2mM of ganciclovir (GCV, Sigma G2536). The medium was changed with SFM with or without 2mM of GCV every two days. Chemicals were supplied by Sigma-Aldrich (Lyon, France), except for glutamine, F12 medium and antibiotics which were supplied by Gibco, Invitrogen (France). For immunolabelling 14 nasal explants were fixed at 10 days in vitro (DIV) with 4% formaldehyde for 15 minutes and stored at 4°C (PBS 0.1% azide) until immunostaining.

Sheep anti-GnRH serum

Immunization

Six lambs received eight intradermic injections and one intravenous injection of a synthetic peptide corresponding to the amino-acid sequence of mammalian GnRH-2-10: HWSYGLRPG, coupled to bovine serum albumin (BSA) with glutaraldehyde (Aneosystem, Groupe NSPE, France) in position 2 (Caraty *et al.*, 1980).

Radioimmuno Assay (RIA)

Cross reactivity of GnRH, GnRH-2, GnRH-Hyp9, GnRH-2-10 and radiolabelled I^{125} GnRH ($GnRH^{125}$, Sigma L7134) on GnRH#517 serum (1:150,000 in glycerolated PBS) was tested as previously described (Caraty *et al.*, 1980). The amino-acid sequence of the GnRH-2 peptide of chicken (Peninsula, 7210) shows 70% similarity with GnRH (supplementary data, Figure S1). It differs primarily in C-terminal moiety by the presence of a tryptophan 7-position and tyrosine at position 8 instead of a leucine and proline respectively (supplementary data, Figure S2). GnRH(HyP9) (Neosystem, SP89180C) presents a hydroxylated proline in position 9. GnRH(2-10) peptide (NeoMPS) contains the sequence of GnRH with a deletion of glutamic acid in position 1 at the N-terminal moiety.

Immunofluorescence

Sample preparation

Six adult male mice were deeply anesthetized with intra-peritoneal administration of sodium pentobarbital (CEVA Santé Animale, France; 200 mg/kg). When inter-costal muscle paralysis occurred, the thoracic cavity was opened and the left ventricle was perfused with 32ml of 1% sodium nitrite in phosphate buffered saline (PBS) followed by 160ml of 4% paraformaldehyde (PAF) in PBS (pH 7.4). The brain was then removed and preserved in 30% sucrose at 4°C. Brains were embedded in Tissue-Tek (Sakura-Fnetek, Europe BV, Zoeterwoude, the Netherlands) and frozen in carbonic ice before sectioning. Three sets of coronal sections (30µm thick) from the preoptic area to the premammillary nucleus were cut on a cryostat (Leica Biosystems CM 3050S, Nanterre, France) and kept in 0.1% sodium azide PBS at 4°C.

Eleven pregnant mice were sacrificed by cervical dislocation; FVB/N-Tg (GFAP-GFP) hemizygous and FVB/N wild type embryos were taken at E11.5, E12.5, E13.5 and E15.5, and GnRH-GFP C57Bl6/j embryos were taken at E11.5 and E15.5. Embryo heads were fixed in PAF4% for 24h at 4°C, preserved in 30% sucrose at 4°C and frozen (Geller *et al.*, 2013). Head sagittal sections (20µm) were mounted on a SuperfrostPlus slide (Thermo FisherScientific Inc., Waltham, MA, USA) and stored at -20°C until immunolabelling.

Immunolabelling

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Lyon, France). Secondary antibodies used are listed in Table 1.

Adult hypothalamic sections: free-floating coronal sections from adult GnRH-GFP C57BL6/j homozygous (n=3) and FVB/N wild type (n=3) mice were incubated for 20 minutes at room temperature (RT) in 2% bovine serum albumin in PBS 0.3% Triton X-100 (PBS-BSA2). The first set of sections of each brain were incubated with sheep anti-GnRH #517 (1:10,000 in PBS-BSA2) overnight at 4°C. After three PBS rinses, sections were then incubated in secondary antibody for 2h30 at RT.

Embryos: head sections (from n=28 embryos) were pre-incubated in 10% bovine serum albumin PBS 0.3% Triton X-100 (PBS-BSA10).

Simple and double labelling with sheep anti-GnRH #517 (1:1,000) and chicken anti-GFP, (1:1,000, Abcam) were performed on E15.5 wild type FVB/N (n=2) and E15.5 homozygous GnRH-GFP C75Bl6/j (n=2). Triple labelling for BLBP (Rabbit anti-BLBP, 1:1,000, Millipore), GnRH and GFP were performed on FVB/N-Tg (GFAP-GFP) hemizygous embryos at E12.5 E13.5 E15.5 (n=12). Head sections were incubated in primary antibodies overnight at 4°C. After washing three times with PBS, sections were incubated in secondary antibody for 2h30 at RT. For GnRH-GFP embryos, GFP antibody was labelled with FITC anti-chicken IgY secondary antibody (1:500, Jackson Immunoresearch). For GFAP-GFP embryos, GFP signals were amplified using biotinylated anti-chicken IgY (1:500, Vector, for 2h at RT), and Streptavidine DyLight-488 (1:1,000, Jackson Immunoresearch, for 1h at RT).

Triple labelling for BLBP and Peripherin and GnRH or GFP, and double labelling for BLBP and $p75^{NTR}$ or Aldh1L1 (aldehyde dehydrogenase 1 family) were performed following the procedure described by (Negoescu *et al.*, 1994). Sections from FVB/N wild type embryos (at E12.5 E13.5 E15.5, n=9) and from GnRH-GFP homozygous embryos (at E11.5, n=4) were incubated in anti-GnRH #517 and anti-GFP, respectively. They were incubated in rabbit anti-BLBP overnight at 4°C. After three PBS washes, sections were incubated in secondary antibodies for 2h30 at RT. Sections were washed five times in PBS and blocked in normal rabbit serum 1:500 in PBS-BSA10 for 30 minutes at RT. After five PBS washes, saturation of rabbit IgG-globulins was performed with Fab antibody fragments (1:500 in PBS and 0.3% Triton X-100, Jackson ImmunoResearch) for 1h at RT. After five PBS washes, sections were incubated in rabbit anti-peripherin (1:1,500, Millipore), rabbit anti-$P75^{NTR}$ (1:500, Promega) or rabbit anti-Aldh1L1 (1:1,000, Millipore) overnight at 4°C. Sections were washed three times in PBS and incubated in secondary antibody for 2h30 at RT. Nasal explant immunolabelling was performed as previously described (Geller et al., 2013). Briefly the explants were incubated for 20 minutes in PBS-BSA10 at RT and incubate overnight at 4°C in rabbit anti-GFAP (1:400, Dako) and mouse anti-GnRH (1:5,000, Abcam [SMI 41]) diluted in PBS-BSA10. After washing three times, secondary antibodies were incubated for 2h at RT.

After the immunolabelling procedure, all sections and nasal explants were incubated for 2 min in 2 µg/ml Hoechst 33258 (Molecular Probes, Invitrogen) to label nuclei. Sections and explants were then mounted in Fluoromount (Southern Biotech, Birmingham, Alabama, USA).

Controls

Immunolabelling specificity was confirmed by omitting the primary antibody and the disappearance of cellular labelling. The labelling specificity of the sheep anti-GnRH #517 was tested by an overnight pre-adsorption of the primary antibody (1:10,000 for adult sections or 1:1,000 for embryonic sections) in the presence of 25 or 250µM GnRH peptide (Sigma, L7134). Immunohistochemistry was performed as previously described on FVB/N wild type adult mouse brain sections and on head sections from FVB/N wild type and GnRH-GFP embryos aged from E10.5 to E15.5. Labelling with the sheep anti-GnRH #517 antibody was detected at E12.5, no labelling was detected at E10.5 or E11.5.

Image analysis

All observations and acquisitions were carried out on a laser confocal microscope (Zeiss LSM 700) using ZEN software. 1024x1024 stacks (z=0.5 or 1µm) were acquired sequentially using a x40 objective. 512x512 mosaics were acquired using a x20 objective.

In situ quantitative analysis: for each GnRH+ cell, the nucleus was centred in the x,y and z plans (objective x40, field surface 155µm^2). Cells were defined as "associated to GnRH neurones" if the distance between cell nuclei and GnRH nuclei was equal to or less than twice the average radius of glial nuclei (i.e., ≤ 6.05µm). Measurements and counts were performed with ZEN software.

Ex vivo measurement analysis: to examine the whole culture (including inside and outside the explant), mosaic images were acquired with a confocal microscope. The projection length of GnRH extensions was obtained by measuring the distance between the placode exit and GnRH+ fibre ends which were furthest from the nasal explant using ZEN software.

Statistical analysis

For *in situ* analysis statistical tests were performed using Prism 4.0 software (GraphPad Software Inc., La Jolla, USA) and for *ex vivo* analysis statistical tests were performed using R (*http://www.R-project.org*). Normality was tested using the Shapiro-Wilk normality test. Comparison between ages (E12.5, E13.5 and E15.5) and regions (NS, NJF) of the distribution between BLPB+, [GFAP-GFP+] and double-labelled BLBP/[GFAP-GFP+] cells were performed with two way ANOVA, after square root transformation of data using a mixed model two way analysis of variance. Post hoc comparisons were performed with Bonferroni adjustment. For comparisons of projection length of GnRH extensions, the data did not fit a normal distribution, comparison between groups was therefore performed using a Man-Whitney rank test. Mean and SEM (standard error of mean) are reported in the results section.

Results

In order to characterize the phenotypic development of the glial microenvironment of migrating GnRH neurones in the AOS, BLBP were used as a marker of pOEC and [GFAP] (using GFAP-GFP transgenic mice) as a marker of mOEC. To demonstrate the benefits of using these markers, labelling against Aldh1L1 (marker of precursor and mature glial cells) and $p75^{NTR}$ (mOEC marker) was also performed (see supplementary data, Figure S1). A new antibody against GnRH in sheep (GnRH#517) was also developed to allow triple immunolabelling. The antibody was characterize using RIA and immunohistochemistry in adult brain slices and embryonic head slices of mice (see supplementary data, Figures S2 –S3 –S4).

Characterization of the BLBP progenitor microenvironment of migrating GnRH neurones

Existing data on the distribution of pOEC BLBP+ cells associated with migrating GnRH neurones is poor. We therefore initially investigated the spatiotemporal evolution of BLBP+ labelling along peripherin axons within the AOS, telencephalon and diencephalon of embryos at E11.5 (n=4), E12.5 (n=6), E13.5 (n=8) and E15.5 (n=7). For the E11.5 stage,

GnRH-GFP embryos were used because no GnRH immunoreactivity could be detected with the anti-GnRH#517.

At E11.5 the majority of GnRH-GFP+ were located within or in the vicinity of the VNO (Figure 1A). Unlike GnRH neurones located in the VNO (red arrow, Figure 1B), GnRH neurones located outside the VNO were with BLBP+ cells (Figure 1B). BLBP immunoreactivity was weak and presented fibrillary labelling in the nasal septum, no labelling was observed in the VNO (Figure 1B). At E12.5, BLBP+ cells were closely associated with GnRH+ neurones along the peripherin+ axons from their VNO exit (Figure 1C, D) to the ventral part of the olfactory bulb (OB) called the nasal forebrain junction (NFJ) (Figure 1C, E). At higher magnification we observed that BLBP+ formed a sheath around GnRH+ cells in NS (Figure 1D1). At the NFJ, BLBP+ cells formed a migratory cellular mass around GnRH+ neurones (Figure 1E1). We also observed BLBP+ elongated cells at the distal part of the telencephalon (Figure 1 C, F). These BLBP+ cell bodies were present along the peripherin+ axons and had a fusiform morphology, similar to the BLBP labelling in the peripheral AOS (Figure 1F3 *versus* Figure 1D1). In this area, GnRH+ neurone cell bodies and neurites were also associated with these BLBP+ cell bodies (arrow, Figure 1F3) and BLBP+ fibres (arrow head, Figure 1F3). At E13.5 and E15.5, BLBP+ cells were detected along the peripherin+ axons in the NS (at the VNO exit) at the NFJ (along axons in the ventral part of OB) and in the diencephalon (E13.5 data not shown and Figure 1G). At E13.5 there was a close association between GnRH+ and BLBP soma and fibres with their cytoplasmic processes in NS and NFJ (data not shown). At E15.5 BLBP+ cells formed a sheath around GnRH+ neurones in the NS, and at NFJ (arrows, Figure 1G). At this stage, we also observed BLBP+ fusiform cells along the peripherin+ axons and in the vicinity of GnRH+ neurones in the diencephalon (arrow, Figure 1H).

Thus, migrating GnRH neurones were found to be closely associated with BLBP+ pOEC cells at the VNO exit from E11.5, in the NS, NFJ and telencephalon between E12.5 and E15.5 and also in the diencephalon at E15.5.

Spatio-temporal characterisation of the OEC maturation in the accessory olfactory system along the migratory pathway of GnRH neurones.

Secondly, we studied the glial cell maturation in the AOS along the migration route of GnRH neurones. We focused our analysis on i) the rostral olfactory migratory pathway delimited by the VNO and the cribriform plate of the ethmoid, designated in this study as the nasal septum region (NS) and ii) the transition zone between the nasal septum and the diencephalon where most GnRH neurones turned ventro-caudally to migrate tangentially towards the preoptic and hypothalamic areas. This area encompassed the NFJ and the OB and is referred to here as the NFJ-OB. We quantified the proportion of mOEC [GFAP-GFP+], pOEC BLBP+ and doubly labelled BLBP+/[GFAP-GFP+] on GnRH neurones in 155µm^2 fields (Figures 2A-D) for each migratory territory, NS and NFJ-OB at E12.5 (n=3), E13.5 (n=5) and E15.5 (n=4) (Figure 2E). Note that [GFAP-GFP+] was detected only at E13.5.

In the NS, the proportion of single labelled BLBP+ cells decreased between E12.5 and E13.5 and E13.5 and E15.5 (ANOVA ***$F_{2,18}$=23.19 p=0.00001***, respectively by -30.8%, ***t=4.659 p=0.001***, and -25.40% non-significantly ***t=2.485 p=0.138***), whereas the proportion of BLBP+/[GFAP-GFP+] cells increased (ANOVA ***$F_{2,18}$=16.48 p=0.000086,*** respectively by +20.2% ***t=3.271 p=0.0254,*** and +26.5%, non-significantly ***t=2.871 p=0.0608***). The proportion of [GFAP-GFP+] cells increased (ANOVA ***$F_{2,18}$=9.280 p=0.0017***) between E12.5 and E13.5 (by +10.65%, ***t=3.769 p=0.008439***), but not between E13.5 and E15.5 (10.65±3.9% versus 9.5±3.1%, ***t=0.331 p>0.99***). In the NFJ-OB, no significant difference was found between developmental stages regardless of the marker. Thus, the proportion of single labelled BLBP+ cells differed significantly between NFJ-OB and NS (ANOVA ***$F_{1,9}$=20.01 p=0.0015***) at E13.5 (92.8±3.6% versus 69.1±7.9%, ***t=3.745 p=0.013***) and E15.5 (84±8.2% versus 43.7±6%, ***t=4.568 p=0.004***). The proportion of double labelled BLBP+/[GFAP-GFP+] cells was also significantly smaller in the NFJ-OB than with NS (ANOVA ***$F_{1,9}$=22.60 p=0.0010***) at E13.5 (2.7±1.5% versus 20.2±6.7%, ***t=3.829 p=0.012***) and E15.5 (13±8.3% versus 46.7±6.3%, ***t=4.991 p=0.0022***).

To summarize, in the NS, OEC phenotype changed from progenitor to mature as embryonic age increased. This development was not statistically significant at the NFJ-OB where pOEC represented the major OEC population at any embryonic stage.

Spatio-temporal characterisation of cells associated with migrating GnRH neurones

To test whether GnRH neurones were also preferentially associated with pOEC or mOEC depending on the region crossed, we performed the same spatiotemporal analysis but restricted it to cells located less than 6.05µm from GnRH neurone nuclei (white arrow, Figure 3A), and including BLBP+ cells (red arrows), BLBP+/[GFAP-GFP+] cells (yellow arrows) and [GFAP-GFP+] cells. GFP, BLBP and GnRH immunolabelling was performed on GFAP-GFP transgenic mouse embryos at E12.5 (n=3), E13.5 (n=5) E15.5 (n=4) (Figure 3).

Between E12.5 and E15.5 there was a significant difference in the proportion of BLBP+ cells and double-labelled BLBP+/[GFAP-GFP+] according to the embryonic stage (respectively two-way ANOVA ***$F_{2,18}$=16.37 p=0.000088*** and two-way ANOVA ***$F_{2,18}$=20.61 p=0.000022***) and their localization (NS or NFJ-OB, respectively two-way ANOVA ***$F_{1,9}$=8.808 p=0.0157*** and two-way ANOVA ***$F_{1,9}$=26.8028 p=0.00058***). We detected an interaction between the two factors (respectively two-way ANOVA *$F_{2,18}$=3.9345 p=0.038* and two-way ANOVA *$F_{2,18}$=4.9450 p=0.01942*) suggesting that development of the glial cell phenotype seems to depend on their anatomic localization. Bonferroni post-hoc comparison between embryonic ages in NS showed that the proportion of BLBP+ cells associated with GnRH neurones decreased significantly between E12.5 (100 %), E13.5 (77.4±7.6%) and E15.5 (46.7±6.5%) **(ab *t=3.346 p=0.021*, ac *t=5.938 p=0.00007*, bc *t=3118 p=0.03*)**; whereas the proportion of BLBP+/[GFAP-GFP+] cells increased between E12.5 and E13.5, and E13.5 and E15.5 **(respectively by 15% *t=3.3021 p=0.023*, and by 31.45% *t=4.038 p=0.0046*)**. In the NFJ-OB, no significant difference was found between developmental stages regardless of the marker. Thus, when comparing anatomical areas the proportion of single-labelled BLBP+ was higher in NFJ-OB than in NS at E15.5 (89.8±5% versus 46.6±6.5%, ***t=3.90 p=0.010***), while no significant difference was detected at E13.5 (91±3.65% versus 77.4±7.65%, ***t=1.511 p=0.494***). The proportion of double-labelled BLBP+/[GFAP-GFP+] cells was also significantly different between NS and NFJ-OB at E13.5 (14.97±4.67% versus 3.3±1.7%, ***t=3.125 p=0.036***)

and E15.5 (46.43±5.3% versus 8.34±5.14%, ***t=3.368 p=0.00038***). The proportion of single-labelled [GFAP-GFP+] cells did not change significantly between NS and NFJ-OB at E13.5 (7.6±3.7% versus 5.7±1.8%, ***t=0.25 p>0.99***) or at E15.5 (6.6±2.5% versus 1.8±1.1%, ***t=1.16 p=0.82***).

We examined whether the phenotypic development of glial cells associated with GnRH neurones was correlated with a qualitative and quantitative change in the microenvironment of GnRH neurones. Firstly, we studied whether the proportion of GnRH neurones associated with glial cells changed according to the developmental stage and the region. We quantified the number of GnRH neurones which were associated or not with at least BLBP+ and/ or [GFAP-GFP+] cells (Table 1). On average 91.6 % of migrating GnRH neurones were associated with at least one glial cell. This proportion did not change significantly with increasing embryonic age or localization **(from 86±0.74% for NFJ-OB at E12.5 to 96.3±2.2% for NS at E15.5).** The cellular microenvironment of these GnRH neurones was then studied further (Figure 3C). The cells associated with GnRH neurones (≤ 6.05µm) were classified into three categories: i) glial cells for BLBP+ and/ or [GFAP-GFP+] cells ii) GnRH cells or iii) undetermined cell types (UD cells) for non-immunoreactive cells, detected by Hoechst nuclear labelling. Considering the three developmental stages and the two areas, on average 53.40 % of cells associated with GnRH neurones were glial cells **(from E12.5 to E15.5 in the NS 51.97±5.68%, 52.78±4.69%, 54.59±1.51% respectively and in NFJ-OB 55.37±2.74% 52.78±4.69% 53.40±3.51%)**. On average 14.75% of cells associated with GnRH neurones were other GnRH neurones. This proportion did not change significantly with increasing embryonic age or localization **(from E12.5 to E15.5 in the NS 17.50±4.93%, 14.27±4.54%, 10.93±2.71% respectively and in NFJ-OB 10.71±4.19% 14.27±4.54% 20.82±4.36%)**

To summarize, the majority of migrating GnRH neurones were associated with glial cells (91.6%). In addition, the cellular microenvironment of GnRH neurones did not change according to the region or development stage, since glial cells formed the main cellular microenvironment of these neurones (53.40%). Interestingly, phenotypic development of these associated glial cells changed according to the region, as did the surrounding glial cells. Maturation of OEC associated with GnRH neurones was observed only in the NS,

whereas pOEC represented the major type of glial cells associated with GnRH neurones in NFJ-OB.

The role of [GFAP+] maturing OEC in GnRH neurone development.

To determine the role of specific maturation of OEC in the NS during GnRH neurone migration, we studied the impact of depleting mOEC on GnRH neurone development using nasal explant cultures from GFAP-HSV-Tk transgenic mice. Nasal explant culture is an ideal model to study GnRH neurone migration and also OEC migration from the olfactory placode to the outside of the explant (Forni et al 2013, Geller et al 2013, Raucci et al 2013). The depletion of proliferative [GFAP+] cells was induced by adding antiviral drug ganciclovir (GCV) in the culture medium and the effect was assessed using immunofluorescence. Double-immunolabelling against GFAP and GnRH was performed on nasal explant cultures treated (GCV+, n=8) or not with ganciclovir (GCV-, n=6).

After 10 DIV, the presence of ganciclovir induced a marked reduction in GFAP immunoreactivity detection outside the explant compared to non-treated cultures (Figure 4B versus Figure 4A). In both conditions, we observed GnRH+ immunoreactivity outside the explant (Figure 4A', B'). GnRH+ neuronal networks were superimposed on GFAP+ glial networks in the absence of ganciclovir (Figure 4C-C''), while in the GCV+ condition, the absence of GFAP+ networks was correlated with a decrease in GnRH+ network expansion (Figure 4A'' versus 4B'') and an increase in GnRH+ network disorganization (Figure 4D-D''). These GnRH+ networks included GnRH+ cell bodies and fibres detected outside the explant in both conditions (Figure 4C'D' and Figure 5AB). On quantifying the number of GnRH+ cell bodies detected outside the explant no statistical difference between GCV+ and GCV- conditions was observed (15±0.58 versus 14.75±2.72, ***p=0.68*** Figure 5C). The distance between the placode exit and the GnRH+ cell bodies furthest from the nasal explant was measured. The maximal distance travelled by these neurones was not significantly different between GCV+ and GCV- (6.8±0.51µm versus 7.37±0.89µm ***p>0.99*** Figure 5D). Focusing on GnRH+ fibres, in the GCV- condition GnRH+ fibres were detected at the level of the cellular migration front, an area characterized by a poor cellular environment as reflected by a low number of nuclei (Figure 6A). However, under the GCV+ condition, GnRH+ fibres were detected mid-way, in a denser cellular environment (Figure 6B). For each culture, we

measured the distance between the placode exit and GnRH+ fibres the furthest from the nasal explant (Figure 6C). The GnRH+ fibre outgrowth decreased by 57.4% in nasal explant cultures treated with ganciclovir compare to non-treated cultures (836.12±96.3µm versus 1917.67±251.5µm, ***p=0.00066***).

To summarize, as expected the presence of ganciclovir induced depletion of GFAP+ OEC, but did not seem to impact GnRH neurogenesis or initiation of GnRH neurone migration in nasal explant cultures. However, the depletion of GFAP+ glial cells induced a significant decrease in the extension of GnRH+ fibres, which could play a role in reducing the expansion and disorganization of the GnRH+ network observed.

Discussion

Maturation of glial cells from their progenitor to mature state is associated with a change of markers in the central and peripheral nervous systems (Kriegstein & Alvarez-Buylla, 2009; Rowitch & Kriegstein, 2010). It has also been shown that the degree of maturation varies according to the localization of the cells within the main olfactory system (Ramon-Cueto & Avila, 1998; Guerout *et al.*, 2010; Su & He, 2010) and within the accessory olfactory system (supplementary data, Figure S1). Thus, the antigenic phenotype exhibited by OEC is dependent on both the developmental stage and the anatomical localization of the cells within the main olfactory system. In the present study, we focused on the OEC within the accessory olfactory system and their association with developing GnRH neurons.

The BLBP progenitor OEC colonize the AOS from the VNO exit to the telencephalon and seem to be present in the diencephalon during embryonic development.

We determined that OEC progenitors accompanied GnRH neurones along the vomeronasal nerve pathway as early as E11.5; crossing the main olfactory bulbs medially and projecting to the accessory olfactory bulbs. So, our results show that the developmental pattern of OEC progenitors on the migratory pathway of GnRH neurones in the accessory olfactory system was correlated with the developmental pattern of OEC progenitors cells in the main olfactory system (Anthony *et al.*, 2004; Carson *et al.*, 2006; Murdoch & Roskams, 2007; Miller *et al.*, 2010; Blanchart *et al.*, 2011).

We also detected OEC progenitors within the telencephalic vesicle (presumptive olfactory bulb) along olfactory axons suggesting that these cells enter the central nervous system. Previous immunohistochemical and electron microscope studies have already demonstrated the presence of OEC in the telencephalic region throughout embryonic development and also in adulthood (Marin-Padilla & Amieva, 1989; Valverde *et al.*, 1992; Lopez-Mascaraque *et al.*, 1996). Also, Zheng & Jourdan (Zheng *et al.*, 1988; 1988) highlighted glial cells ensheathing terminal nerve axons from the olfactory bulb surface and ventro-caudally at the level of the terminal ganglion in adult rats. The present study detected BLBP+ cells along terminal nerve axon peripherin+ from the olfactory bulb region at E12.5 to the diencephalon at E15.5. Additional studies would be required to determine whether these BLBP+ cells were progenitors of central glial cells or OEC progenitors. However, *in vitro* studies have demonstrated that the degree of OEC coupling through gap junctions can regulate GnRH neuronal activity and secretion (Pinet-Charvet *et al.*, 2016).

OEC mature in the nasal septum of AOS during GnRH neurones migration: a rostro-caudal maturation similar to the main olfactory system.

Although we detected OEC progenitors along all the migratory pathways of GnRH neurones in accessory olfactory system, mature GFAP-GFP cells were mostly present in the proximal part of this pathway. When focusing on the close microenvironment of GnRH neurones, we determined the same characteristics for glial cells associated with GnRH neurons. Interestingly, despite these glial phenotypic differences, the proportion of glial cells associated with GnRH neurones remained stable (≥ 64%) whatever the developmental stage or the region crossed. Overall, these data suggest that the glial phenotype developed from progenitors to mature OEC in the nasal septum, while progenitors remained the main OEC population at the nasal forebrain junction and at the olfactory bulb level. This change in glial phenotype was previously described in the main olfactory system, and was also considered as a maturation process of OEC (see for review Ramon-Cueto & Avila, 1998). The developmental pattern of GFAP expressing cells on the migratory pathway of GnRH neurones is correlated with the developmental pattern of GFAP immunoreactivity in the main olfactory system (Valverde *et al.*, 1992; Astic *et al.*, 1998; Wang *et al.*, 2008; Blanchart

et al., 2011). There is a time and space dependent rostro-caudal differentiation of OEC from E12.5 to E15.5.

To account for the difference in OEC phenotype distribution between nasal septum and nasal forebrain junction areas, we hypothesized that mature OEC would not be able to move and only progenitor OEC could migrate from the nasal septum to the nasal forebrain, olfactory bulb area. Indeed, different OEC subpopulations have been shown to exhibit remarkable differences in their motility within the main olfactory system (Rowitch & Kriegstein, 2010; Su & He, 2010) (Marin-Padilla & Amieva, 1989; Valverde *et al.*, 1992). BLBP expression itself could stimulate cellular migration since down-regulation of BLBP expression by small interfering RNAs significantly reduced cell migration in neurospheres and in gliostoma cell cultures (De Rosa *et al.*, 2012; Schnell *et al.*, 2014). In contrast, GFAP is an intermediate filament conferring a greater rigidity to the cytoskeleton (Astic *et al.*, 1998). Mature OEC would slow down and stop migrating along the GnRH migration pathway, while BLBP+ OEC progenitors would migrate with GnRH neurones into the brain.

[GFAP+] mature OEC are involve in GnRH neurite outgrowth: a distinct role of mature OEC and progenitors OEC in GnRH neurone development.

The difference in spatial distribution of mature OEC and OEC progenitors during GnRH neurone nasal migration raises the question of the role of these glial cell types in the development of GnRH neurons. Recently, the role of OEC progenitors in GnRH neurone development has been described (Barraud *et al.*, 2013; Pingault *et al.*, 2013), thus raising our interest in the role of mature OEC in GnRH neurone development. In this study, we observed that the depletion of GFAP+ cells in nasal explant cultures caused a decrease of 57.4% in GnRH neurite outgrowth, suggesting mature OEC play a role in the morphological differentiation of GnRH neurones. Migrating GnRH neurones grew and became bipolar between E12.5 and E15.5 (Schwanzel-Fukuda & Pfaff, 1989; Wray *et al.*, 1989; Fueshko & Wray, 1994). This morphological differentiation has been correlated with maturation of GnRH peptide and its detection in the trans golgi network (Wray, 2002). Thus, the differentiation of GnRH neurones took place at the same developmental stages as the increase in numbers of [GFAP+] mature OEC in the nasal septum. All these data are

consistent with our observations showing that mature OEC induce neurite outgrowth of migrating GnRH neurones and suggest for the first time that glial cells are key players in the differentiation of GnRH neurones.

The importance of glial cells in neuronal development is well documented in the central and peripheral nervous system, but regarding GnRH neurones, the majority of studies have focused on the involvement of glial cells in peri-pubertal development of the GnRH network and its regulation in adulthood (Sharif *et al.*, 2013). Glial cell involvement in embryonic and perinatal development of GnRH neurones is a recent field of investigation and only a few studies have developed this subject. In fact two recent studies showed that loss of function of the *Sox10* gene led to complete absence of mature Schwann cells, impaired OEC differentiation and defective GnRH neurone migration to the brain in the mouse and suggest the importance of BLBP+ OEC progenitors in GnRH neurone migration (Barraud *et al.*, 2013; Pingault *et al.*, 2013). *In vitro* depletion of [GFAP+] mature OEC did not impact GnRH neurogenesis or migration but only affected GnRH neurite outgrowth. All these data suggest that OECs are involved in different processes of GnRH neurone ontogenesis according to their maturation level. Thus, the diversity of OEC phenotypes observed may explain the diversity of phenotypes observed in hypogonadotropic hypogonadism patients, as suggested by Forni et Wray (2015) . It is known that in the CNS the effects of glial cells on neuronal development differ according to their phenotype (Powell *et al.*, 1997; Rakic, 2003). Similarly, in the main olfactory system, *in vitro* and *in vivo* data from several studies have indicated that OEC can act differently in neuronal development according to their phenotypes (Kafitz & Greer, 1999; Wewetzer *et al.*, 2001; Lipson *et al.*, 2003; Zhang *et al.*, 2008; Su & He, 2010; Jiao *et al.*, 2011; Sethi *et al.*, 2014). Mature OEC expressing the neurotrophin receptor $p75^{NTR}$ have been shown to increase neurite outgrowth significantly in brain slices (Jiao *et al.*, 2011). It has also been suggested that mature OEC expressing $p75^{NTR}$ increased the total neurite length of cortical neurones in culture by providing both an adhesive cellular substrate and permissive soluble factors (Khankan *et al.*, 2016). In line with our research, this latter study demonstrated a correlation between OEC process alignment with neurites and the size of neurite elongation, and it suggested that OEC process alignment was a method for OEC to enhanced neurite elongation/outgrowth.

Conclusion

Our study shows a rostro-caudal maturation of olfactory ensheathing glial cells in the accessory olfactory system and suggests that according to their maturation stage OECs play a distinct role in GnRH neurone development. Our results demonstrate that mature OEC are involved in neurite outgrowth of GnRH neurones and suggests that unlike OEC progenitors, mature OEC play a role in morphological differentiation of GnRH neurones but not in their migration.

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Conflict of interest

Authors have no conflict of interest to declare.

Author contribution

SG and PV designed experiments; SG, DL, AC, PV conducted experiments; SG, AHD, PV performed data analyses; SG, YT, AHD and PV contributed to the discussion. SG and PV supervised the project and edited the manuscript.

Data Accessibility

All primary data are available on demand from PV and SG.

Abbreviations

Aldh1L1: aldehyde dehydrogenase 1 family

AOS: accessory olfactory system

BLBP: brain lipid binding protein

CNS: central nervous system

DIV: days *in vitro*

GCV: ganciclovir

GFAP: glial fibrillary acid protein

GFP: green fluorescent protein

GnRH: gonadotropin-releasing hormone

mOEC: mature OEC

NFJ: nasal forebrain junction

NGFR : nerve growth factor receptor

NS: nasal septum

OB: olfactory bulbs

OEC: olfactory ensheathing cells

OP: olfactory placode

PNS: peripheral nervous system

pOEC: OEC progenitors

$p75^{NTR}$: p75 neurotrophin receptor

VNO: vomeronasal organ

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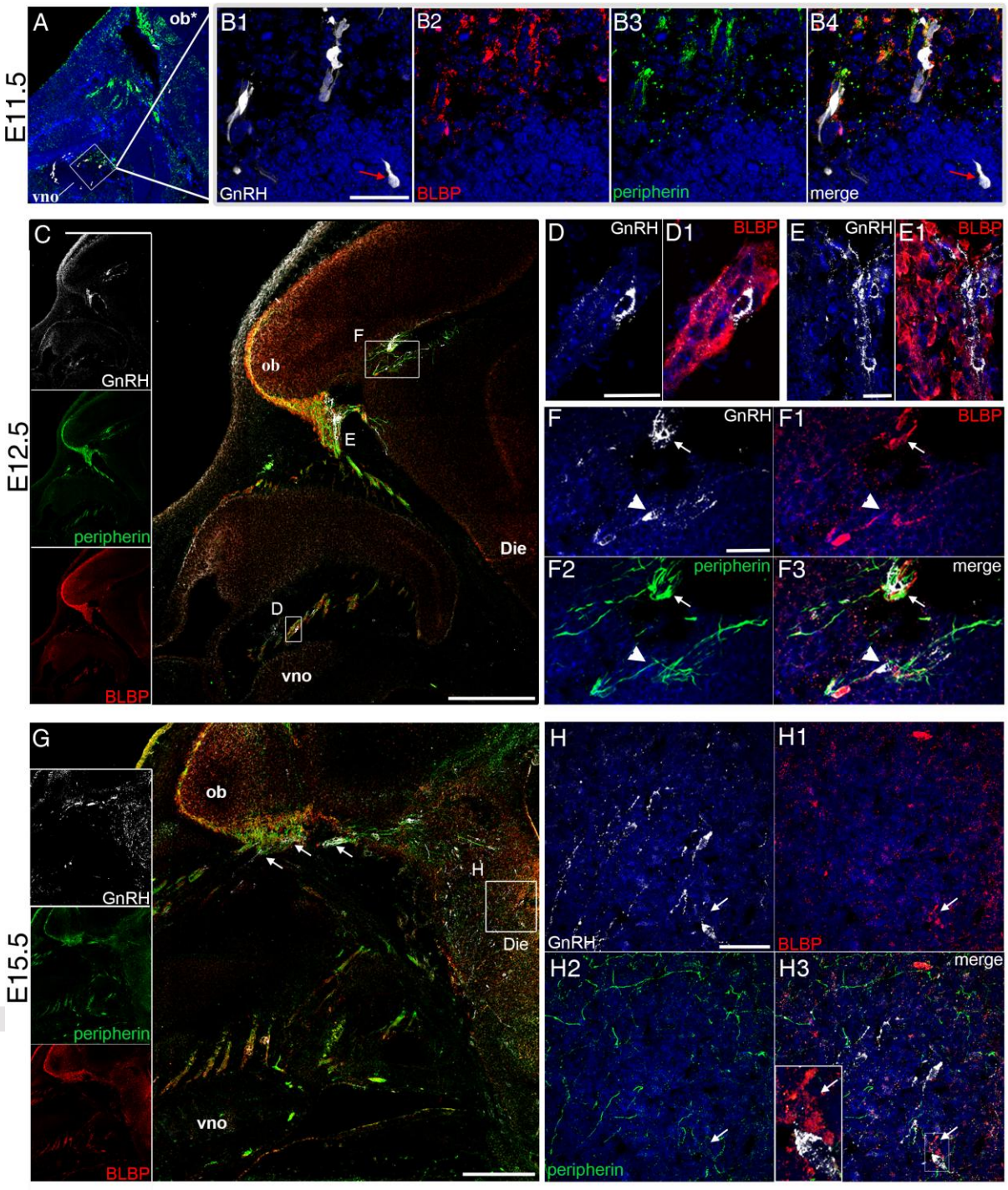
Table 1: Secondary antibodies for antigen revelation.

Secondary antibodies	Source	Dilution
CY3 anti-sheep IgG (H+L)	Jackson ImmunoResaearch	1:500
FITC anti-chicken IgY	Jackson ImmunoResaearch	1:500
Alexa 647 anti-rabbit IgG	Molecular Probes	1:1,000
Alexa 555 anti-rabbit IgG	Molecular Probes	1:1,000
Alexa 488 anti-Rabbit IgG	Molecular Probes	1:1,000

Table 2: The proportion of GnRH neurones associated with at least one glial cell (BLBP+ and/or [GFAP-GFP+] cells) in NS and NFJ-OB at E12. 5 (n = 3), E13. 5 (n = 5) and E15. 5 (n = 4).

Regions	Nasal Septum (NS)			Nasal forebrain junction (NFJ-OB)		
Embryonic development stages	E12.5	E13.5	E15.5	E12.5	E13.5	E15.5
% of GnRH neurones associated with at least one glial cell	90.3±5.7	91±6.03	96.3±2.2	86±0.74	93±4.5	93±1.6



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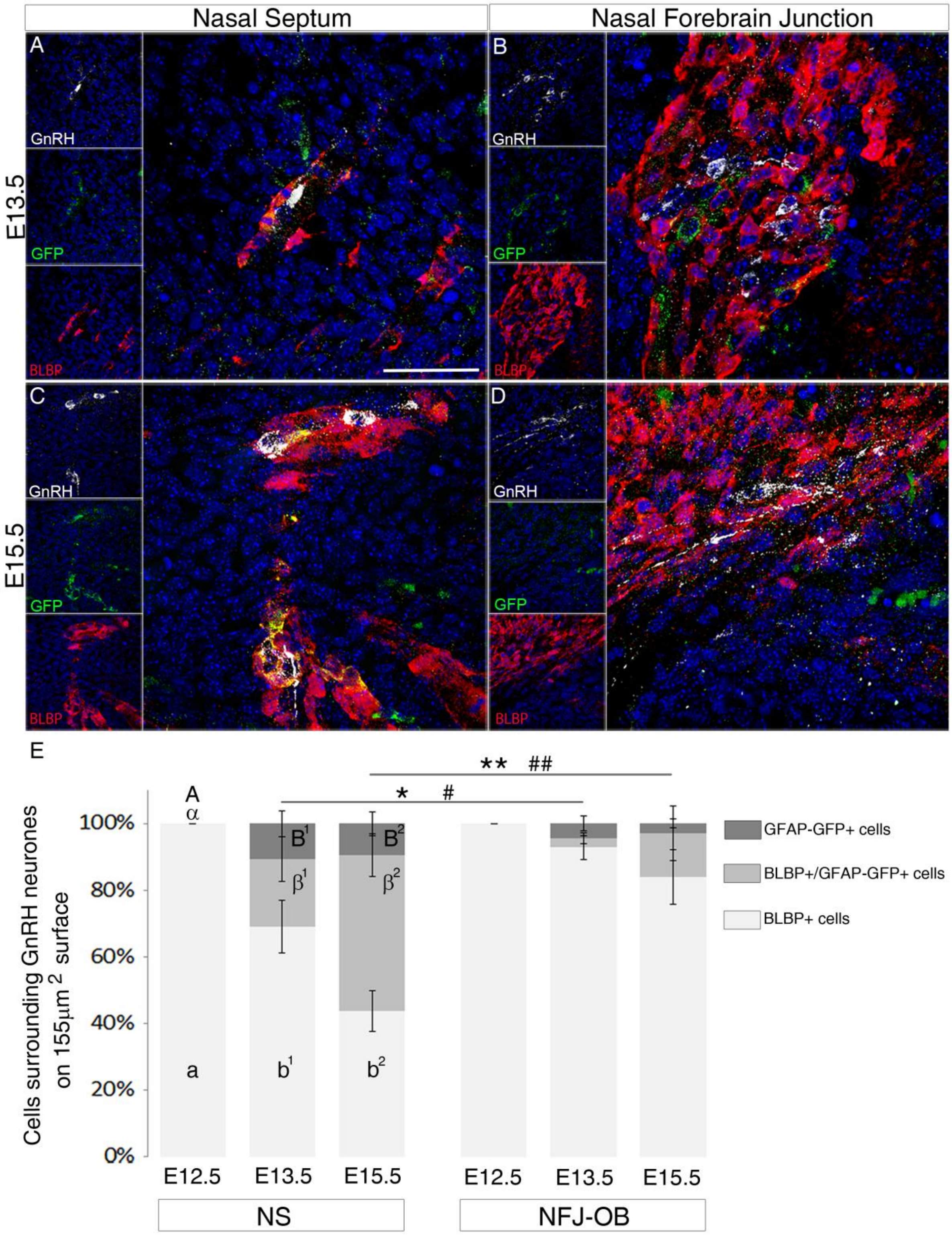

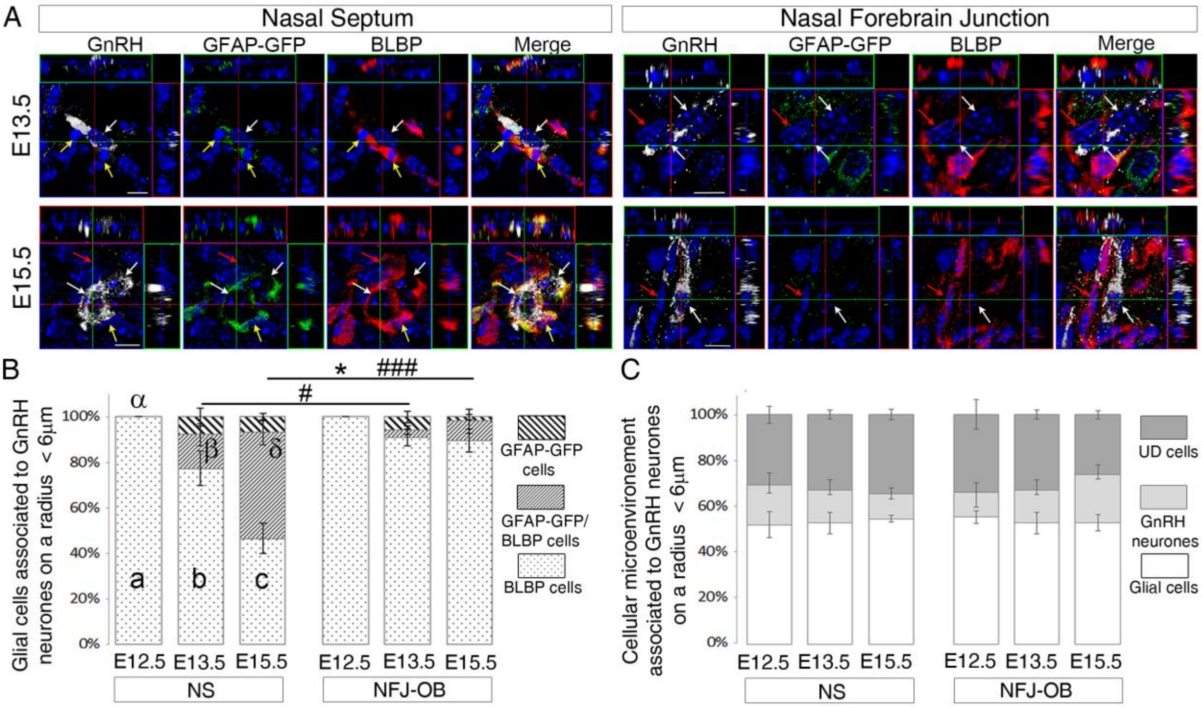
A
Nasal Septum
Nasal Forebrain Junction
GnRH
GFAP-GFP
BLBP
Merge
E13.5
E15.5
B
Glial cells associated to GnRH neurones on a radius < 6µm
100%
80%
60%
40%
20%
0%
α
β
δ
a
b
c
* ###
#
E12.5
E13.5
E15.5
NS
NFJ-OB
GFAP-GFP cells
GFAP-GFP/ BLBP cells
BLBP cells
C
Cellular microenvironement associated to GnRH neurones on a radius < 6µm
UD cells
GnRH neurones
Glial cells