Progressive Effects of *N-myc* Deficiency on Proliferation, Neurogenesis, and Morphogenesis in the Olfactory Epithelium

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ABSTRACT: *N-myc* belongs to the *myc* proto-oncogene family, which is involved in numerous cellular processes such as proliferation, growth, apoptosis, and differentiation. Conditional deletion of *N-myc* in the mouse nervous system disrupted brain development, indicating that *N-myc* plays an essential role during neural development. How the development of the olfactory epithelium and neurogenesis within are affected by the loss of *N-myc* has, however, not been determined. To address these issues, we examined an *N-myc^{Foxg1Cre}* conditional mouse line, in which *N-myc* is depleted in the olfactory epithelium. First changes in *N-myc* mutants were detected at E11.5, with reduced proliferation and neurogenesis in a slightly smaller olfactory epithelium. The phenotype was more pronounced at E13.5, with a complete lack of *Hes5*-positive progenitor cells, decreased proliferation, and neurogenesis. In addition, stereological analyses revealed reduced cell size of post-mitotic neurons in the olfactory epithelium, which contributed to a smaller olfactory pit. Furthermore, we observed diminished proliferation and neurogenesis also in the vomeronasal organ, which likewise was reduced in size. In addition, the generation of gonadotropin-releasing hormone neurons was severely reduced in *N-myc* mutants. Thus, diminished neurogenesis and proliferation in combination with smaller neurons might explain the morphological defects in the *N-myc* depleted olfactory structures. Moreover, our results suggest an important role for *N-myc* in regulating ongoing neurogenesis, in part by maintaining the *Hes5*-positive progenitor pool. In summary, our results provide evidence that *N-myc* deficiency in the olfactory epithelium progressively diminishes proliferation and neurogenesis with negative consequences at structural and cellular levels.   © 2013 The Authors. Developmental Neurobiology Published by Wiley Periodicals, Inc. Develop Neurobiol 74: 643–656, 2014

Keywords: neurogenesis; *N-myc*; olfactory epithelium; vomeronasal organ; mouse

INTRODUCTION

The *myc* family of proto-oncogenes consists of *c-myc*, *N-myc*, and *L-myc*, three related genes involved in diverse biological processes such as proliferation, differentiation, and apoptosis [reviewed in (Henriksson and Luscher, 1996; Facchini and Penn, 1998; Eilers and Eisenman, 2008)]. Mice deficient in *c-myc* or *N-myc* die at about embryonic day 10 (E10) or E12, respectively (Charron et al., 1992; Trumpp et al., 2001), whereas *L-myc* deficiency has no lethal phenotype (Hatton et al., 1996). *N-myc*-deficient embryos display delayed development of organs,

which normally express high levels of *N-myc*, such as the heart, lung, and gut (Charron et al., 1992). Interestingly, these developmental defects in *N-myc* mutants occurred despite the compensatory increase of *c-myc* expression (Stanton et al., 1992), which underlines the essential functions of *N-myc* during development. Conditional deletion of *N-myc* in neuronal progenitor cells prevented the early lethal phenotype and uncovered *N-myc* as crucial factor during development of the nervous system (Knoepfler et al., 2002; Dominguez-Frutos et al., 2011; Kopecky et al., 2011). *N-myc*-deficient mice exhibited abnormal behavior in correlation with a twofold reduction in brain mass, including severe defects in the cerebellum (Knoepfler et al., 2002). In addition, conditional deletion of *N-m*yc in the otic placode severely affects inner ear development, including perturbed morphology and disorganized neuronal innervation (Dominguez-Frutos et al., 2011; Kopecky et al., 2011).

As part of the peripheral nervous system, the olfactory placode gives rise to the olfactory epithelium (also known as main olfactory epithelium) and the vomeronasal organ. In general, olfactory sensory neurons in the olfactory epithelium transmit odor sensation, while the vomeronasal organ detects pheromones and both structures project axons to discrete target regions of the olfactory bulb. In addition, gonadotropin-releasing hormone (GnRH) neurons have been suggested to originate from the epithelia of the vomeronasal organ and medial wall of the olfactory pit (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). The GnRH neurons migrate in association with olfactory epithelial- and vomeronasal organ-derived axons towards the hypothalamus in the forebrain (Wray et al., 1994; Norgren et al., 1995; Yoshida et al., 1995). The production of GnRH in the hypothalamus controls the reproductive system in vertebrates by stimulating the release of gonadotrophins from the anterior pituitary, which affects the gonadal functions [reviewed in (Wray, 2010)]. To date, no correlation between N-myc and GnRH neurons has been reported.

The olfactory epithelium is one of the few regions in the nervous system, where neurogenesis persists throughout life to generate olfactory sensory neurons. Therefore, it serves as a useful model system to study regulatory processes during embryonic and adult neurogenesis (Cau et al., 1997; Kawauchi et al., 2009; Tucker et al. 2010; Fletcher et al., 2011; Maier et al., 2011; Packard et al., 2011). Neurogenesis in the olfactory epithelium begins already at olfactory placodal stages in both mouse and chick, and the sensory neuronal lineage contains stem-like cells, neuronal precursor cells at different maturity stages and

post-mitotic olfactory neurons (Kawauchi et al., 2005; Maier and Gunhaga, 2009; Wei et al., 2013). The different cell types can be defined by the expression of specific molecular markers; stem-like progenitor cells express the basic helix-loop-helix repressor gene *Hes5* (Cau et al., 2000; Maier and Gunhaga, 2009), the immediate neuronal precursor cells are defined by the expression of *Neurogenin1* (*Ngn1*) (Cau et al., 2002; Maier and Gunhaga, 2009), and cells committed to leave the cell cycle express the terminal neuronal differentiation marker *NeuroD1* (Cau et al., 2002). All post-mitotic neurons express the general neuronal markers HuC/D (Fornaro 2003) and Tuj1 (Wei et al., 2013), while a subset of the neurons express the LIM-homeodomain transcription factor Lhx2 (Hirota and Mombaerts, 2004; Kolterud et al., 2004). However, the potential impact of *myc* proto-oncogenes on the development of the olfactory epithelium and neurogenesis therein has not been addressed.

In this study, we have analyzed the influence of *N-myc* on the development of the olfactory epithelium and neurogenesis within, using a recently described conditional mouse line, where *N-myc* deficiency is restricted to *Foxg1*-positive cells (Dominguez-Frutos et al., 2011). The expression of *Foxg1* (also known as *brain factor-1*) is detected already at the initiation of neurogenesis in the olfactory placode of mice at E9.5 (Xuan et al., 1995), and also at later stages of olfactory development (Kawauchi et al., 2009). Our results show a progressive effect of *N-myc* deficiency displaying reduced proliferation, neurogenesis, and disturbed morphogenesis in the mouse olfactory epithelium from E10.5 until E13.5. At E13.5, the population of stem-like progenitors is depleted and proliferation and the generation of neurons are reduced, resulting in a severely reduced olfactory epithelium. Thus, *N-myc* is an essential factor for ongoing neurogenesis and proper development of the olfactory sensory epithelium.

METHODS

Transgenic Mouse Embryos

A recently described conditional *N-myc* transgenic mouse line (Dominguez-Frutos et al., 2011) was used. Briefly, the *N-myc^{Foxg1Cre}* line was generated by crossing *N-myc$^{flox/flox}$* mice (Knoepfler et al., 2002) with a mouse strain carrying a Cre recombinase under control of the *Foxg1* locus (Hebert and McConnell, 2000). *N-myc^{Foxg1Cre}* (hereafter referred to as *N-myc$^{-/-}$*) mutants display *Foxg1*-mediated *loxP* recombination in the telencephalon and discrete head

structures including the olfactory epithelium. Less than 10% of homozygous N-myc mutants are obtained from heterozygous crosses as previously discussed in (Dominguez-Frutos et al., 2011). Both, the *N-myc* and *Foxg1* gene loci are localized on the same chromosome, which leads to the heterozygous loss of the *Foxg1* coding region in *Foxg1-N-myc* mutants (Hebert and McConnell, 2000). This could explain the breeding anomaly of the $N\text{-}myc^{-/-}$ strain in respect to the Mendelian inheritance pattern. The generation and genotyping of the $N\text{-}myc^{-/-}$ mouse line was performed as previously described (Dominguez-Frutos et al., 2011). The embryos were fixed in 4% PFA at 4°C for 3–6 hours, cryoprotected in 30% sucrose at 4°C overnight (ON), embedded in TissueTek (Gibco, Stockholm, Sweden), frozen and stored at −80°C. The use of $N\text{-}myc^{-/-}$ mutant and control mice was approved by the Committee on the Welfare of Experimental Laboratory Animals of the University of Valladolid.

In Situ RNA Hybridization and Immunohistochemistry

In situ RNA hybridization was performed essentially as previously described (Wilkinson and Nieto, 1993) on transversal consecutive sections (10 μm) of the entire olfactory epithelium. Applied mouse digoxigenin-labeled probes were as follows: *c-myc* (Kapeli and Hurlin, 2011), *Hes1* (Apelqvist et al., 1999), *Hes5* (Machold et al., 2007), *Ngn1* (gift from G. Fishell), *NeuroD1* (Cau et al., 1997), *N-myc* (Potvin et al., 2010), and *Notch1* (Stump et al., 2002). Immunohistochemistry was performed using standard protocols. Briefly, sections were blocked in 10% fetal calf serum at room temperature (RT) and primary antibodies were incubated at 4°C ON. Antibodies used were as follows; monoclonal mouse antibodies: anti-HuC/D (1:200, Molecular Probes, Göteborg, Sweden) and anti-neuronal class III Beta-Tubulin (Tuj1) (1:500, Covance, USA), polyclonal rabbit antibodies: anti-phospho-Histone H3 (1:500, Millipore, Solna, Sweden), anti-Lhx2 (1:4000, gift from Thomas M. Jessell), anti-cleaved Caspase3 (1:1000, Cell Signaling, Stockholm, Sweden), and anti-GnRH (1:1000, Fisher, Göteborg, Sweden). Sections were incubated with the appropriate Alexa Fluor secondary antibodies (1:400, Molecular Probes, Göteborg, Sweden) for 1 hour at RT and nuclei were stained using DAPI (1:600, Sigma, Stockholm, Sweden). Slides were mounted with fluorescent or Glycergel mounting medium (Dako, Stockholm, Sweden).

Stereology

The size of HuC/D$^+$ (HuC/D-positive) neurons was analyzed using the unbiased estimation of the volume of particles (Gundersen, 1986). Images of the olfactory epithelium and vomeronasal organ were taken using a 0.75 numerical aperture lens on a Nikon Eclipse E800 microscope, equipped with a CCD camera connected to a PC (Nikon Imaging Software NIS-Elements). Images of HuC/D and DAPI staining were merged and processed with Photoshop CS2 software (Adobe) and the diameter of 25–30 neurons per structure and hemisphere was measured using ImageJ software (http://rsb.info.nih.gov/ij/). The unbiased volume of HuC/D$^+$ neurons was calculated with the following estimation; $\pi/3$ times of the diameter length l_0 raised to the third power (Gundersen, 1986).

Statistical Analysis and Imaging

The quantification of Caspase3, HuC/D, Lhx2, pHH3, and GnRH immunopositive cells as well as *Hes5, Ngn1*, and *NeuroD1 in situ* positive cells were performed using a 0.75 numerical aperture lens on a Nikon Eclipse E800 microscope. At all stages used in this study, the left and right hemispheres were analyzed separately and the mean values used for statistics. In addition, at E11.5 and E13.5, the medial and lateral parts of the olfactory epithelium were analyzed separately. All quantitative data of cell numbers and cell size were compared between age-matched $N\text{-}myc^{-/-}$ mice and control littermates. To consider the reduced morphology of the olfactory epithelium and vomeronasal organ in $N\text{-}myc^{-/-}$ embryos, the total number of cells was determined by counting the number of DAPI-positive nuclei. All data from the cell counting were corrected to the total cell number by structure and hemisphere. Quantification and image generation was performed using a Nikon Eclipse E800 microscope for simultaneous Epi-fluorescence/DIC observations, equipped with a CCD camera connected to a PC (Nikon Imaging Software NIS-Elements). Images were processed using Photoshop CS2 (Adobe). The graphs represent the mean number or mean ± SEM if not stated otherwise. Significant effects were confirmed by *Student's t* test, with *p* values of <0.05 (*), <0.01 (**), <0.0001 (***) accepted as statistically significant.

RESULTS

N-myc is Expressed at the Onset of Neurogenesis in the Olfactory Placode

To examine whether *N-myc* is expressed at the onset of neurogenesis in the olfactory placode, we analyzed the expression of *N-myc* and various markers of the sensory neuronal lineage in E9.5 wild-type mouse embryos. *N-myc* expression was scattered throughout the olfactory placode at E9.5, and consistent with the onset of neurogenesis cells in the olfactory placode also expressed *Hes5, Ngn1, NeuroD1*, HuC/D, and Tuj1 (Fig. 1). However, Lhx2$^+$ post-mitotic neurons were not generated at E9.5 (Fig. 1). Proliferative cells were detected in the olfactory placode indicated by expression of phosphorylated Histone H3 (pHH3), a marker for mitotic cells (Sholl-Franco et al., 2010) (Fig. 1). Thus, the transcription factor *N-myc* is expressed at the onset of neurogenesis in the olfactory placode of mice.
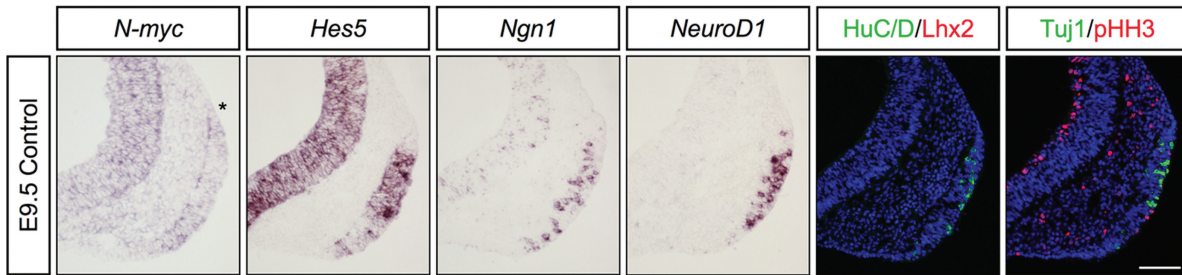
Figure 1 N-myc is expressed at E9.5, at the onset of neurogenesis, in the olfactory placode. At E9.5, cells in the mouse olfactory placode express the neurogenic markers *N-myc, Hes5, Ngn1*, and *NeuroD1*. HuC/D$^+$ post-mitotic neurons, Tuj1$^+$ neurons, and pHH3$^+$ proliferative cells are detectable in the olfactory placode, but not Lhx2$^+$ post-mitotic neurons. The borders of the olfactory placode are indicated by asterisks. Scale bar: 100 μm.

Neurogenesis in the Olfactory Epithelium is not Dependent on *N-myc* at E10.5

To study the influence of *N-myc* on early neurogenesis in the olfactory epithelium, we analyzed E10.5 *N-myc*$^{-/-}$ mice and their control littermates. As expected, *N-myc* expression was present in the olfactory epithelium of control embryos, and was completely absent in *N-myc*$^{-/-}$ embryos at E10.5 [Fig. 2(A)]. However, no differences in the generation of *Hes5*$^+$ stem-like progenitors and *Ngn1*$^+$ neuronal precursors were detectable between *N-myc*$^{-/-}$ and control mice [Fig. 2(A)]. Double labeling with HuC/D and Lhx2 revealed the onset of Lhx2 expression in a subset of post-mitotic neurons in the olfactory epithelium at E10.5 [Fig. 2(A)]. The generation of Lhx2$^+$ neurons, Tuj1$^+$ neurons was unaffected in *N-myc* mutants, and the quantitative analysis of HuC/D$^+$ neurons and proliferative pHH3$^+$ cells revealed no differences between *N-myc*$^{-/-}$ mice and their control littermates [Fig. 2(A–C)]. These data indicate that at E10.5, early neurogenesis in the olfactory epithelium is not affected by *N-myc* deficiency.

To evaluate whether a possible up-regulation of *c-myc* expression could compensate for the loss of N-myc activity at this early stage, we analyzed *c-myc* expression in the olfactory epithelium at E10.5. However, no difference in *c-myc* expression was detected in the olfactory epithelium of *N-myc*$^{-/-}$ mice at E10.5 compared to control embryos (Supporting Information Fig. 1).

Altered Neurogenesis and Morphological Defects Observed in *N-myc*$^{-/-}$ Mice at E11.5

Next, we examined the olfactory epithelium in *N-myc*$^{-/-}$ mice and their control littermates at E11.5.

Consistent with the results from E10.5, *N-myc* expression in the olfactory epithelium was only detected in control mice, but not in E11.5 *N-myc*$^{-/-}$ embryos [Fig. 3(A)]. In addition, the generation of *Hes5*$^+$ stem-like progenitors, *Ngn1*$^+$ neural precursors, and *NeuroD1*$^+$ neuronal differentiation was reduced in the olfactory epithelium of *N-myc*$^{-/-}$ mutants [Fig. 3(A)].

Interestingly, the olfactory epithelium was smaller in *N-myc*$^{-/-}$ embryos, exhibiting a less prominent prospective vomeronasal organ in comparison to control animals [Fig. 3(A)]. This morphological defect was not caused by increased apoptosis as Caspase3 expression was not changed (data not shown). In contrast, the number of HuC/D$^+$ post-mitotic neurons was reduced in both the medial and lateral part of the *N-myc*-deficient olfactory epithelium [Fig. 3(B)]. In addition, the number of Lhx2$^+$ neurons was also reduced in the medial and lateral part of the olfactory epithelium in *N-myc*$^{-/-}$ embryos, to a similar extent as HuC/D$^+$ neurons (Supporting Information Fig. S2). Moreover, proliferation indicated by pHH3 expression was also reduced, however, only in the medial part of the *N-myc*-deficient olfactory epithelium [Fig. 3(C)]. Double labeling with HuC/D and Lhx2 indicated that approximately 90% of HuC/D$^+$ post-mitotic neurons also expressed Lhx2 in both *N-myc*$^{-/-}$ and wild-type embryos [Fig. 3(B)].

Both proliferation and *Hes5* expression has been shown to be regulated by Notch activity (Ohtsuka et al., 1999; Basak and Taylor, 2007). Subsequently, we also examined the expression of *Notch1* in the olfactory epithelium. At E11.5, *Notch1* expression was clearly reduced in the olfactory epithelium of *N-myc*$^{-/-}$ mice [Fig. 3(A)], indicating that *N-myc* activity is required for Notch activity and proliferation in the olfactory epithelium. Thus, at E11.5 loss of *N-myc* reduces Notch activity, proliferation, and ongoing neurogenesis, resulting in a smaller olfactory epithelium.
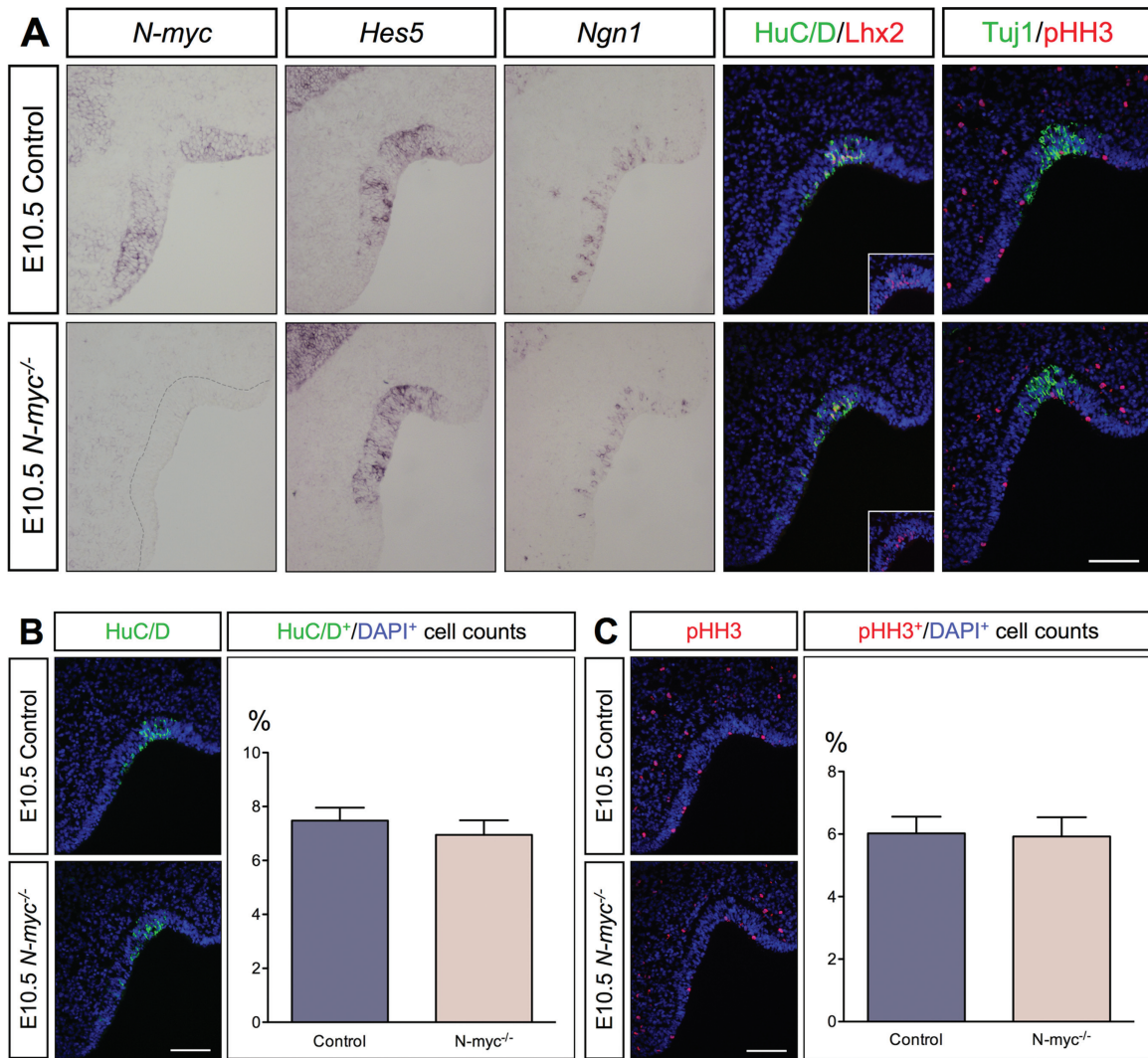
Figure 2 At E10.5, the development of the olfactory epithelium is not dependent on *N-myc*. (A) The expression of *N-myc* is completely missing in the olfactory epithelium of E10.5 *N-myc* mutants, whereas the generation of $Hes5^+$ stem-like progenitors and $Ngn1^+$ neuronal precursors are not different between both genotypes. The generation of $Lhx2^+$, HuC/D^+, and $Tuj1^+$ post-mitotic neurons are not different between mutants and controls. The onset of Lhx2 expression is indicated in the insets. (B,C) *N-myc* deficiency did not alter the number of HuC/D^+ post-mitotic neurons (B) or proliferation, indicated by $pHH3^+$ staining (C). Statistical analysis of the cell counts in comparison to the total cell number in E10.5 control embryos ($n = 5$) and $N-myc^{-/-}$ animals ($n = 4$) (HuC/D $p = 0.5296$, pHH3 $p = 0.9104$). Error bars represent $\pm$SEM, Student's *t* test. Scale bars: 100 μm.

Progressive Effects on Olfactory Neurogenesis in *N-myc*$^{-/-}$ Mutants

To cover a later phase of olfactory neurogenesis, we investigated E13.5 mouse embryos, where the vomeronasal organ has separated from the olfactory epithelium. Despite the normal appearance of the mouse head, especially the nose, the olfactory epithelium was severely reduced in size in E13.5 $N-myc^{-/-}$ mutants [Fig. 4(A)]. Important to note is that apoptosis was not the cause for the smaller olfactory epithelium, since the number of Caspase3$^+$ cells was similar in $N-myc^{-/-}$ compared to wild-type mice (Supporting Information Fig. S3A). In contrast, the number of pHH3$^+$ mitotic cells was reduced in both the medial and lateral part of the *N-myc*-deficient olfactory epithelium [Fig. 4(C)]. Thus, the differential loss in proliferation between the
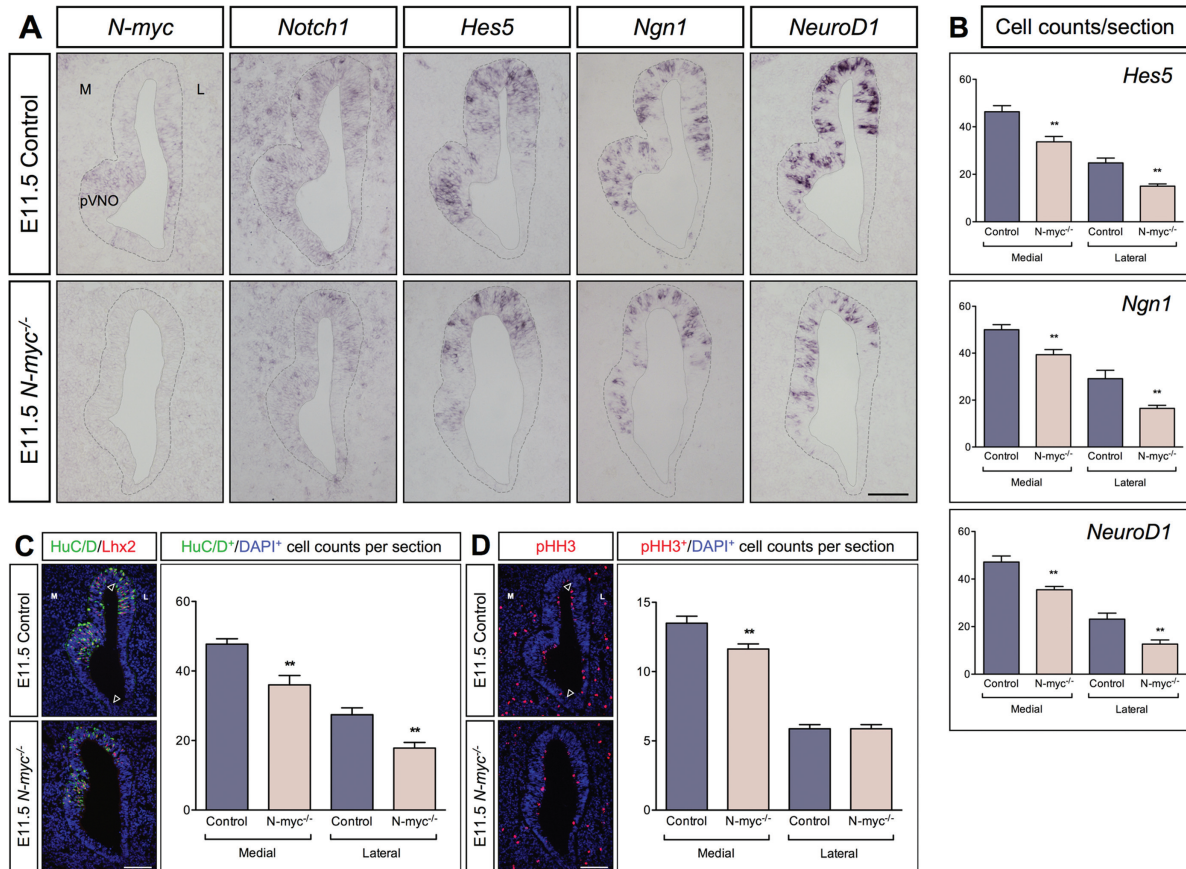
Figure 3 Altered proliferation, neurogenesis, and morphology in the olfactory epithelium at E11.5. (A) *N-myc* expression is completely missing in the olfactory epithelium of E11.5 N-$myc^{-/-}$ mice, *Notch1* expression is down-regulated and the generation of $Hes5^{+}$ progenitors, $Ngn1^{+}$ neuronal precursors, and $NeuroD1^{+}$ cells are decreased compared to controls. At this stage, the morphology of the olfactory epithelium is disturbed, displaying a less prominent area of the prospective vomeronasal organ (pVNO) in mutant mice. (C,D) The number of HuC/D^{+} and $Lhx2^{+}$ postmitotic neurons (C), and the number of $pHH3^{+}$ mitotic cells are reduced (D). The borders between the medial (M) and lateral (L) part of the olfactory epithelium are indicated by arrowheads. (B–D) Statistical analysis of the cell counts in comparison to the total cell number defined by DAPI in the E11.5 olfactory epithelium of controls ($n = 7$) and N-$myc^{-/-}$ embryos ($n = 6$) for *Hes5* (control versus N-$myc^{-/-}$ medial **$p = 0.0041$, and lateral **$p = 0.0013$), *Ngn1* (control versus N-$myc^{-/-}$ medial **$p = 0.0060$, and lateral **$p = 0.0071$), *NeuroD1* (control versus N-$myc^{-/-}$ medial **$p = 0.0023$, and lateral **$p = 0.0062$), HuC/D (control versus N-$myc^{-/-}$ medial **$p = 0.0025$, and lateral **$p = 0.0037$), and pHH3 (control versus N-$myc^{-/-}$ medial, $n = 8$, **$p = 0.0096$). Error bars represent $\pm$SEM, Student's *t* test. Scale bars: $100 = \mu m$.

medial and lateral parts observed in the olfactory epithelium of *N-myc* mutants at E11.5 were no longer detectable at E13.5

Interestingly, at E13.5 the generation of $Hes5^{+}$ stem-like progenitor cells was completely lost [Fig. 4(A)], without any compensatory up-regulation of *Hes1* (Supporting Information Fig. S3B). Consistently, *Notch1* expression was diminished in *N-myc*-deficient mice [Fig. 4(A)]. Furthermore, the generation of $Ngn1^{+}$ neural precursors and $NeuroD1^{+}$ terminally

differentiated neurons appeared to be reduced in N-$myc^{-/-}$ embryos [Fig. 4(A)]. Consistently, the number of HuC/D^{+} neurons was reduced in both the medial and lateral part of the *N-myc*-deficient olfactory epithelium [Fig. 4(B)]. Our data at E13.5 indicate a progressive effect of *N-myc* deficiency, in which decreased proliferation, reduced Notch activity and depletion of the $Hes5^{+}$ progenitor cells results in a significantly smaller olfactory pit and suppressed neurogenesis.
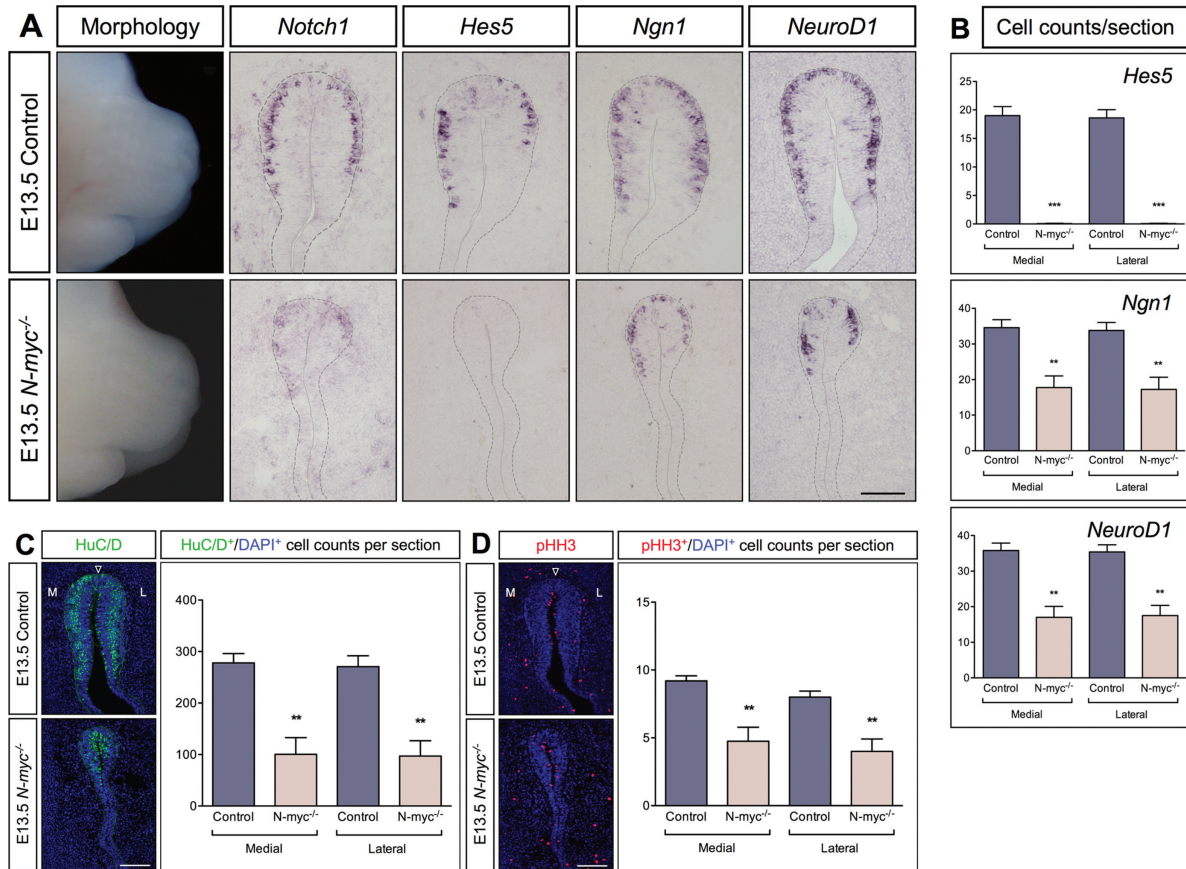
Figure 4 Progressive reductions of proliferation, neurogenesis, and morphology in the olfactory epithelium at E13.5. (A) The nose is indistinguishable between *N-myc* mutants and control embryos. However, *Notch1* expression was reduced, the generation of $Hes5^+$ progenitors was completely diminished, and the olfactory epithelium was malformed and severely reduced in size in *N-myc* mutants. Moreover, the generation of $Ngn1^+$ neuronal precursors, and $NeuroD1^+$ cells decreased in *N-myc* mutants. (C,D) The number of HuC/D^+ post-mitotic neurons (C) and proliferative $pHH3^+$ cells (D) are reduced in the medial (M) and lateral (L) part of the olfactory epithelium. (B–D) Statistical analysis of the cell counts in comparison to the total cell number in the olfactory epithelium of E13.5 controls ($n = 5$) and *N-myc* mutants ($n = 4$) for *Hes5* (control versus $N\text{-}myc^{-/-}$ medial ***$p \leq 0.0001$, and lateral ***$p \leq 0.0001$), *Ngn1* (control versus $N\text{-}myc^{-/-}$ medial **$p = 0.0032$, and lateral **$p = 0.0040$), *NeuroD1* (control versus $N\text{-}myc^{-/-}$ medial **$p = 0.0012$, and lateral **$p = 0.0011$), HuC/D (control versus $N\text{-}myc^{-/-}$ medial **$p = 0.0015$, and lateral **$p = 0.0018$), and pHH3 (control versus $N\text{-}myc^{-/-}$ medial **$p = 0.0030$, and lateral **$p = 0.0040$). Error bars represent $\pm$SEM, Student's *t* test. Scale bars: 100 μm.

Development of the Vomeronasal Organ and GnRH Neurons are Dependent on *N-myc*

At E11.5, the vomeronasal organ is part of the olfactory epithelium, but separates from it around E13 to develop independently. The severe effect of *N-myc* deficiency on the E13.5 olfactory epithelium guided us to also analyze the vomeronasal organ in these embryos. We detected a much smaller vomeronasal organ in $N\text{-}myc^{-/-}$ mutants compared to control littermates (Fig. 5). The decreased size of the vomeronasal organ was not due to an increase in apoptosis, as measured by the presence of Caspase3$^+$ cells (data not shown). The generation of $Ngn1^+$ neural precursors and terminal neuronal differentiation indicated by *NeuroD1* was strongly reduced in *N-myc*-deficient mice [Fig. 5(A)]. Furthermore, the number of HuC/D^+ post-mitotic neurons was reduced in both the sensory and non-sensory part of the vomeronasal organ [Fig. 5(B)], whereas a reduction in proliferative $pHH3^+$ cells was restricted to the sensory portion of
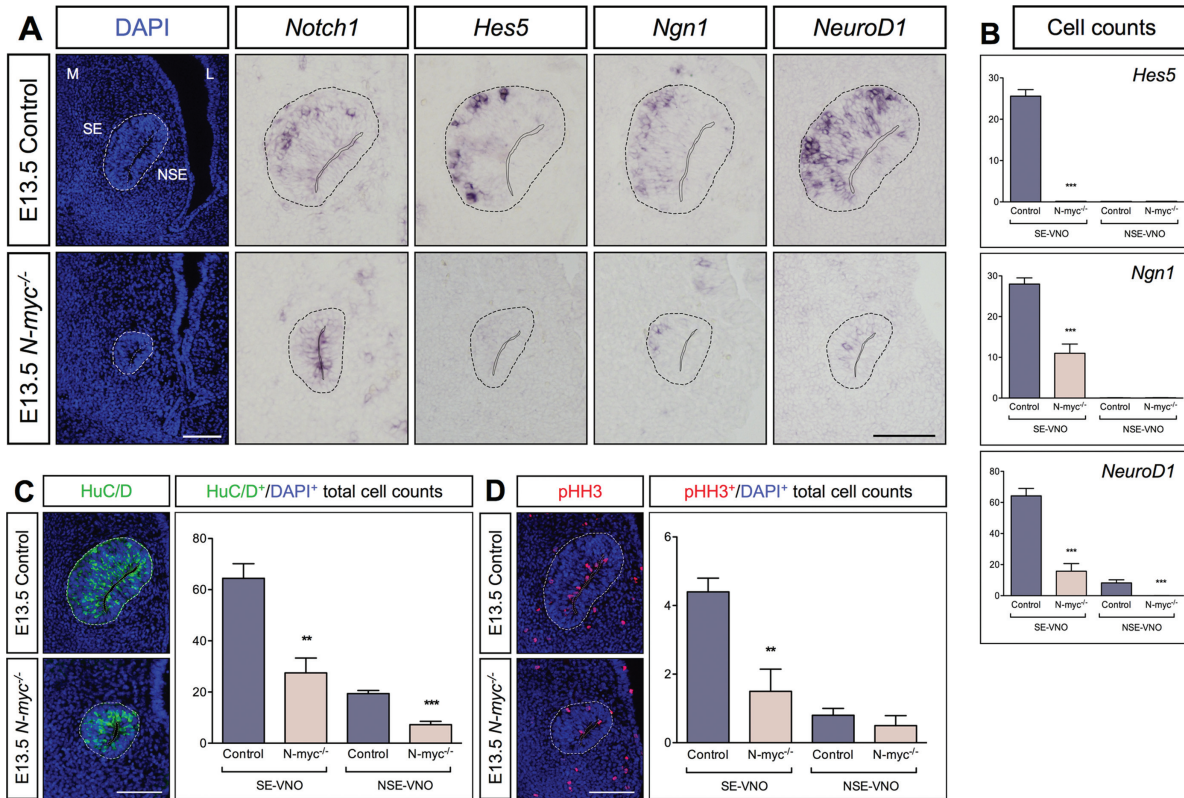
Figure 5 Development of the vomeronasal organ is dependent on *N-myc*. (A) The vomeronasal organ (VNO) is clearly smaller in *N-myc*-deficient mice compared to control animals. The expression of *Notch1* is mildly down-regulated, whereas the generation of $Hes5^+$ stem-like progenitors is completely absent, and $Ngn1^+$ neuronal precursors and $NeuroD1^+$ neurons are reduced in the *N-myc*-deficient VNO. The medial (M) and lateral (L) area of the VNO are indicated. (C,D) The number of HuC/D^+ post-mitotic neurons is reduced in the sensory (SE) and non-sensory (NSE) part (C), while $pHH3^+$ proliferative cells are only reduced in the SE-VNO (D). (B–D) Statistical analysis of the total cell counts in the VNO of E13.5 controls ($n = 5$) and *N-myc* mutants ($n = 4$) for *Hes5* (control versus $N\text{-}myc^{-/-}$ SE-VNO ***$p \leq 0.0001$), *Ngn1* (control versus $N\text{-}myc^{-/-}$ SE-VNO ***$p = 0.0002$), *NeuroD1* (control versus $N\text{-}myc^{-/-}$ SE-VNO ***$p = 0.0003$, and NSE-VNO ***$p \leq 0.0001$), HuC/D (control versus $N\text{-}myc^{-/-}$ SE-VNO **$p = 0.0028$, and NSE-VNO ***$p = 0.0003$) (C), and pHH3 (control versus $N\text{-}myc^{-/-}$ SE-VNO **$p = 0.0052$) (D). Error bars represent $\pm$SEM, Student's *t* test. Scale bars: 100 μm.

the vomeronasal organ [Fig. 5(C)]. Although, $Hes5^+$ stem-like progenitors were absent [Fig. 5(A)], *Notch1* expression appeared to be less affected by *N-myc* deficiency in the vomeronasal organ compared to the olfactory epithelium [Fig. 5(A)]. No compensatory up-regulation of Hes1 expression was observed in *N-myc* mutants (Supporting Information Fig. S3B).

It has been suggested that GnRH neurons originate from the epithelia of the vomeronasal organ and medial wall of the olfactory pit (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). Since both of these structures are severely reduced in size in the *N-myc* mutants [Figs. 4(A) and 5(A)], we analyzed whether the generation of GnRH neurons were

disturbed in *N-myc* mutants at E13.5. Our results show an approximate 85% reduction of GnRH neurons in *N-myc* mutants at E13.5 compared to wild-type embryos (Fig. 6). These results indicate that *N-myc* plays an important role for the development of the vomeronasal organ including the generation of GnRH neurons.

N-myc is Important for Neurons to Maintain Their Cell Size

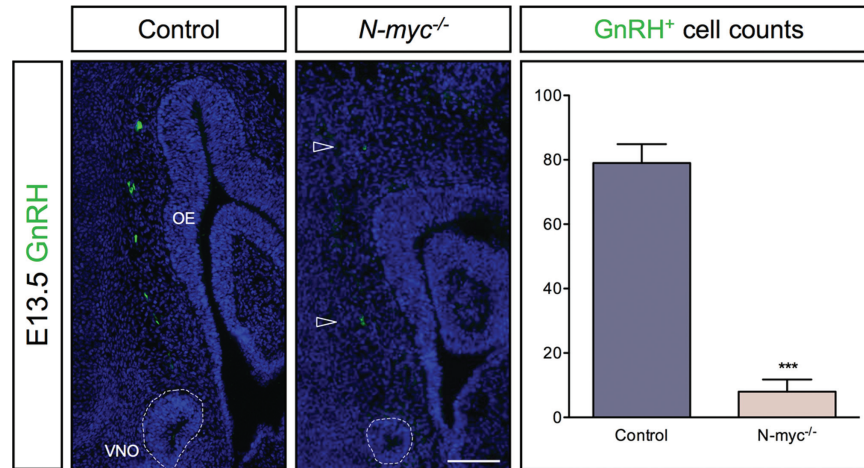To further examine the smaller olfactory pit and vomeronasal organ in *N-myc*-deficient mice, we analyzed

Figure 6 Severely reduced numbers of GnRH neurons in -*myc*-deficient mice. At E13.5, the numbers of GnRH neurons are severely reduced (∼85%) (arrowheads) in *N-myc*-deficient mice (*n* = 3) compared to wild type embryos (*n* = 4) (***p = 0.0002). The olfactory epithelium (OE) and vomeronasal organ (VNO) are indicated. Error bars represent ±SEM, Student's *t* test. Scale bar: 100 μm.

the size of single cells in these structures. HuC/D staining is mainly restricted to the cytoplasm of postmitotic neurons (Fornaro et al., 2003), which are representative for a large subpopulation of cells in the olfactory epithelium. Therefore, HuC/D^+ neurons provided a sufficient number of cells for stereological analysis in the affected olfactory epithelium of $N\text{-}myc^{-/-}$ mutants. At E10.5, the size of HuC/D^+ neurons was not different between $N\text{-}myc^{-/-}$ and control embryos [Fig. 7(A)]. However, at E11.5 HuC/D^+ neurons were about 18% smaller in the olfactory epithelium of $N\text{-}myc^{-/-}$ mutants, with the smallest neurons located in the lateral part [Fig. 7(B)]. At E13.5, these neurons showed a size reduction of approximately 24% in *N-myc*-deficient mice throughout the olfactory epithelium [Fig. 7(C)].

It is worth to note that in wild-type mice, HuC/D^+ neurons almost doubled their size between E10.5 and E11.5 especially in the medial part [Fig. 7(A,B)], suggesting a period of high cellular activity (Pena et al., 2001). At E13.5, HuC/D^+ neurons in the olfactory epithelium were similar in size in the medial and lateral part [Fig. 7(C)]. Reduction in the size of HuC/D^+ neurons from E11.5 to E13.5 were restricted to the medial part, which suggests a more uniform activity of neurons in the entire olfactory epithelium at E13.5 [Fig. 7(B,C)]. Consistently, we also detected smaller HuC/D^+ neurons in the separated vomeronasal organ of E13.5 $N\text{-}myc^{-/-}$ mutants (data not shown). Thus, reduced neurogenesis and proliferation in combination with smaller HuC/D^+ neurons might explain the progressive reduction in size of the olfactory epithelium and vomeronasal organ in *N-myc*-deficient embryos. In summary, our results indicate that *N-myc* acts as an important factor to maintain cellular activity, proliferation, ongoing neurogenesis, and proper morphogenesis of the olfactory epithelium and vomeronasal organ, including the generation of GnRH neurons.

DISCUSSION

Neurogenesis is the process by which neurons are generated from neural stem cells and progenitors. A few structures in the nervous system maintain neurogenesis throughout life, including the olfactory epithelium belonging to the peripheral nervous system. Neurogenesis in the olfactory epithelium occurs in an ordered manner and specific cell types in the neuronal lineage can be identified by distinct markers (Cau et al., 2002; Beites et al., 2005; Murdoch and Roskams, 2007; Maier and Gunhaga, 2009). In addition, the olfactory epithelium has the potential to recover almost completely after injury [reviewed in (Schwob, 2002)], which makes this structure a valuable model system to study regulatory mechanisms of neurogenesis. Despite expanding knowledge about the control of olfactory neurogenesis (Duggan et al., 2008; Tucker et al., 2010; Gokoffski et al., 2011; Maier et al., 2011; Packard et al., 2011), little has been known how members of the *myc* family influence the development of and neurogenesis in the olfactory epithelium. Now our results provide evidence that *N-myc* is required for normal development of the olfactory epithelium to maintain proliferation, neurogenesis and subsequent morphogenesis of the olfactory pit and the vomeronasal organ.
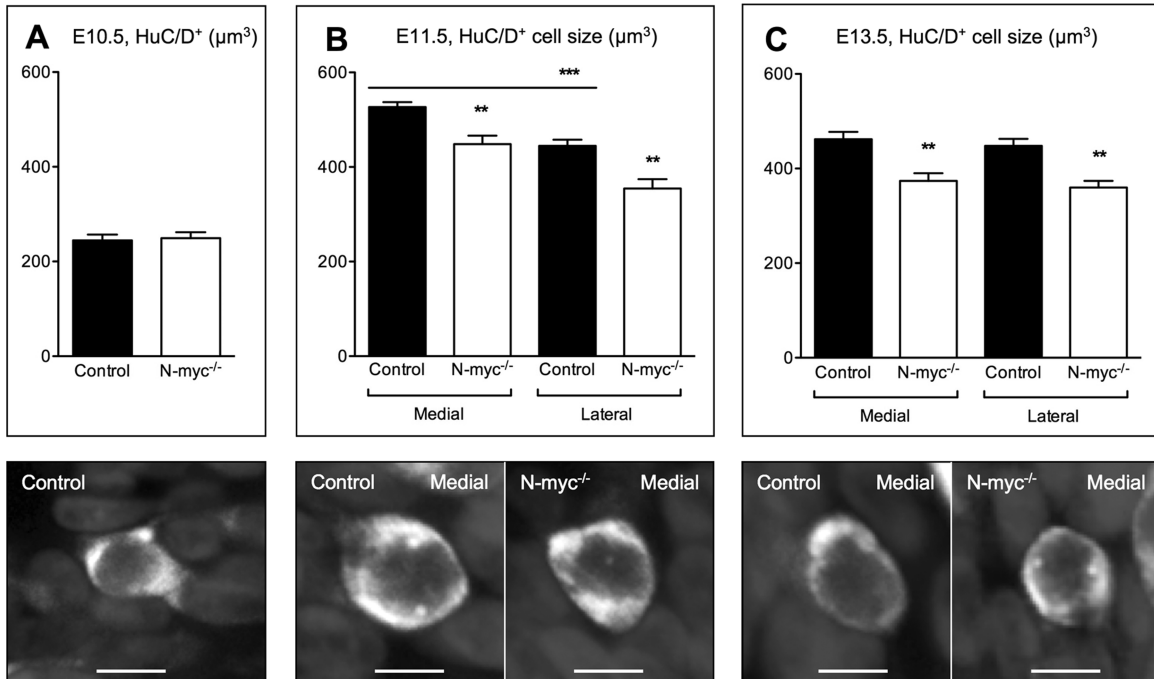
Figure 7 *N-myc*-deficient neurons are smaller in the olfactory epithelium. Stereological analysis of the size of HuC/D$^+$ neurons in the olfactory epithelium at E10.5 (control $n = 5$, *N-myc*$^{-/-}$ $n = 4$), and in the medial and lateral part at E11.5 and E13.5 (E11.5: control $n = 7$, *N-myc*$^{-/-}$ $n = 6$; E13.5: control $n = 5$, *N-myc*$^{-/-}$ $n = 4$). (A) No difference in cell size between E10.5 controls and *N-myc* mutants. (B) At E11.5, HuC/D$^+$ neurons are smaller in the lateral part (medial control versus lateral control ***$p = 0.0003$), and *N-myc*-deficient neurons are smaller in both the medial (**$p = 0.0022$) and lateral part (**$p = 0.0025$) compared to controls. Note that HuC/D$^+$ neurons doubled their size between E10.5 and E11.5. (C) At E13.5, the size of HuC/D$^+$ neurons is similar between the lateral and medial part of controls, but smaller in the medial (**$p = 0.0058$) and lateral part (**$p = 0.0037$) of *N-myc* mutants compared to controls. Error bars represent $\pm$SEM, Student's t test. Scale bars: 5 µm.

Our results show that although *N-myc* is expressed already at E9.5 in the olfactory placode, at the initiation of olfactory neurogenesis, the first changes in *N-myc*-deficient mice are observed somewhat later, at E11.5. At this stage, *N-myc*$^{-/-}$ embryos exhibit reduced neurogenesis in the entire olfactory epithelium, whereas decreased proliferation is only detected in the medial part. These results suggest that the critical role of *N-myc* in the olfactory epithelium is restricted to stages of established neurogenesis. Our finding that *N-myc* is required to maintain proliferation and neurogenesis is in agreement with previous studies suggesting that *N-myc* regulates proliferation and differentiation in the brain, inner ear, and retina (Knoepfler et al., 2002; Martins et al., 2008; Dominguez-Frutos et al., 2011; Kopecky et al., 2011). Consistent with our results, data from the LaMantia lab has also shown that at E11.5 rapid proliferation occurs in the medial part of the olfactory epithelium, while proliferation in the lateral part proceeds in a slow and symmetric manner (Tucker et al., 2010). Interestingly, the rapidly dividing precursor cells in the medial domain of the olfactory epithelium are suggested to give rise to olfactory sensory neurons, vomeronasal neurons, and GnRH neurons (Tucker et al., 2010). Based on results that high levels of *N-myc* expression are present in rapidly cycling progenitors and low levels of *N-myc* in more slowly replicating cells, it has been suggested that *N-myc* regulates the cell cycle in neuronal progenitors (Knoepfler et al., 2002; Wey et al., 2010; Dominguez-Frutos et al., 2011). Thus, it is possible that the higher levels of *N-myc* expression detected in the medial part of the olfactory epithelium define the rapidly dividing neurogenic precursor cells. Furthermore, this might explain why reduced proliferation was only observed in the medial part of the *N-myc*-deficient olfactory epithelium.

At E11.5, the vomeronasal organ is part of the medial olfactory epithelium, and it is not until around E13 that the vomeronasal organ separates and develops independently. In addition, GnRH neurons are first detected at E11 in the medial wall of the developing olfactory epithelium, and later in the separated vomeronasal organ (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). Moreover, it has been shown that GnRH neurons migrate in association with olfactory epithelial- and vomeronasal organ-derived axons towards the hypothalamus in the forebrain (Wray et al., 1994; Norgren et al., 1995; Yoshida et al., 1995). Our analysis of *N-myc*$^{-/-}$ embryos show that already at E11.5, the medial olfactory epithelium is smaller and exhibits a less prominent prospective vomeronasal organ, and at E13.5, the vomeronasal organ is severely reduced in size. Consistently, our results provide evidence that the generation of GnRH neurons are decreased by approximately 85% in E13.5 *N-myc*-deficient embryos. A clinical aspect of a reduced number of GnRH neurons in the hypothalamus is the human Kallmann syndrome (MacColl et al., 2002; Balasubramanian et al., 2010). Patients with Kallmann syndrome suffer from reproductive dysfunction, specifically hypogonadotropic hypogonadism and sometimes also from anosmia (loss of smell). Two genes, *KAL* and *KAL2 (fibroblast growth factor 1 receptor)* have been coupled to a small percentage of Kallmann syndrome cases (Franco et al., 1991; Legouis et al., 1991; Dode et al., 2003). Consequently, the majority of patients with Kallmann's syndrome have mutations in unknown genes, among which our current study has identified *N-myc* as a candidate gene. The embryonic origin of GnRH neuron progenitors has been debated, apart from the olfactory epithelium the adenohypophyseal placode and neural crest cells have also been suggested as possible origins for GnRH neurons in zebrafish (Whitlock et al., 2003). However, in mutant mice with either missing or disrupted anterior pituitaries, GnRH neurons develop normally in association with the vomeronasal organ (Metz and Wray, 2010). Regardless of the origin of the GnRH neurons, our results indicate that a normal development of the medial wall of the olfactory pit and the vomeronasal organ is critical for the generation of GnRH neurons. Whether the reduction of GnRH neurons in *N-myc*$^{-/-}$ embryos are a direct effect caused by the loss of N-myc activity, or a secondary effect due to a disturbed development of the medial wall of the olfactory epithelium and the vomeronasal organ remains to be determined.

Our results show a progressive decrease in proliferation and *Notch1* expression in the *N-myc*-deficient olfactory epithelium, and by E13.5 the *Hes5*$^+$ proliferative pool of cells is completely lost. Consistently, our data also show a progressive reduction in the generation of neurons. Moreover, although the structure of the nose was indistinguishable between wild-type and mutant mice, the morphology of the olfactory epithelium was malformed, and both the olfactory epithelium and the vomeronasal organ were much smaller in size in *N-myc*$^{-/-}$ mice. Our results indicate that the reduction in size of the olfactory structures is not due to increased cell death, but rather caused by decreased proliferation. In addition, our stereological analysis of a distinct population of cells indicated that the generated HuC/D$^+$ neurons are smaller in *N-myc* mutants. However, since the stereological analysis was restricted to HuC/D$^+$ neurons, other cell populations might be also affected. Thus, our results suggest that the reduction of proliferation and neurogenesis, in combination with smaller cell size is responsible for the severe atrophy in both the olfactory epithelium and vomeronasal organ observed in *N-myc*-deficient mice. This hypothesis is in agreement with findings that *myc* genes can activate the expression of several genes that are involved in the regulation of cell size, protein synthesis, and growth (Iritani and Eisenman, 1999; Coller et al., 2000; Boon et al., 2001). Consistently, a previous study suggested that a decrease in cell size might explain the smaller brain of *N-myc*-deficient mice (Knoepfler et al., 2002).

While *N-myc* has been shown to be widely expressed in the nervous system, *c-myc* expression is confined to other tissues and organs (Stanton et al., 1992). Interestingly, increased *c-myc* expression was detected at E10.5 in the neuroepithelium of *N-myc*-deficient mice (Stanton et al., 1992), indicating a compensatory feed-back loop of *myc* genes in the nervous system. However, the up-regulation of *c-myc* expression was not sufficient to attenuate the severe phenotype of *N-myc* deletion (Stanton et al., 1992). Our data now show that the loss of *N-myc* in the mouse olfactory epithelium does not stimulate an up-regulation of *c-myc* levels. On the other hand, *N-myc* has been shown to rescue the essential role of *c-myc* during embryonic development and compensate most of its functions (Malynn et al., 2000). In conclusion, our data provide evidence that *N-myc* is an essential factor for ongoing proliferation and neurogenesis in the olfactory epithelium, and for proper morphogenesis of the olfactory pit and vomeronasal organ.

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