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Odour enrichment increases adult-born dopaminergic neurons in the mouse olfactory bulb

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Abstract
The olfactory bulb (OB) is the first brain region involved in the processing of olfactory information. In adult mice, the OB is highly plastic, undergoing cellular/molecular dynamic changes that are modulated by sensory experience. Odour deprivation induces down-regulation of tyrosine hydroxylase (TH) expression in OB dopaminergic interneurons located in the glomerular layer (GL), resulting in decreased dopamine in the OB. Although the effect of sensory deprivation is well established, little is known about the influence of odour enrichment on dopaminergic cells. Here we report that prolonged odour enrichment on C57BL/6J strain mice selectively increases TH-immunopositive cells in the GL by nearly 20%. Following odour enrichment on TH–green fluorescent protein (GFP) transgenic mice, in which GFP identified both mature TH-positive cells and putative immature dopaminergic cells expressing TH mRNA but not TH protein, we found a similar 20% increase in GFP-expressing cells, with no changes in the ratio between TH-positive and TH-negative cells. These data suggest that enriched conditions induce an expansion in the whole dopaminergic lineage. Accordingly, by using 5-bromo-2-deoxyuridine injections to label adult-generated cells in the GL of TH–GFP mice, we found an increase in the percentage of 5-bromo-2-deoxyuridine-positive dopaminergic cells in enriched compared with control conditions, whereas no differences were found for calretinin- and calbindin-positive subtypes. Strikingly, the fraction of newborn cells among the dopaminergic population doubled in enriched conditions. On the whole, our results demonstrate that odour enrichment drives increased integration of adult-generated dopaminergic cells that could be critical to adapt the OB circuits to the environmental incoming information.

Introduction
Odour information is transduced by olfactory receptor neurons whose axons project from the olfactory epithelium to the olfactory bulb (OB), where the first steps in olfactory information processing take place. Olfactory receptor neuron axon terminals target the OB glomerular layer (GL), establishing synaptic contacts with the apical dendrite of the mitral/tufted cells, the main OB output neurons that project to the olfactory cortex. In the OB, a large population of GABAergic inhibitory interneurons (INs), mainly located in the GL and granule cell layer, plays a central role in the encoding of olfactory information. Notably, olfactory INs are continuously generated throughout adulthood and their integration in the OB circuits is regulated by experience (Ming & Song, 2011).
In the GL, INs operate at the initial stage of olfactory input, providing rapid and strong olfactory nerve-evoked inhibition to mitral/tufted cells (Shao et al., 2012), and forming interglomerular circuits that inhibit neighbouring glomeruli (Aungst et al., 2003; Shirley et al., 2010). OB glomerular INs are classified into different sub-types based on the expression of specific neurochemical markers, electrophysiological and morphological properties, and the connection networks that they establish (Kosaka & Kosaka, 2005; Parrish-Aungst et al., 2007; Kiyokage et al.,

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2010). Although the precise function of each subtype remains elusive, several pieces of evidence support a role for dopaminergic INs as key glomerular elements in the control of odour detection, discrimination and learning (Cave & Baker, 2009). Based on the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis, it has been estimated that dopaminergic INs represent nearly 10–16% of all GABAergic cells in the GL (De Marchis et al., 2007; Panzanelli et al., 2007; Parrish-Aungst et al., 2007). They receive olfactory nerve input either directly through monosynaptic connection or mediated by external tufted cells and/or periglomerular cells (Kiyokage et al., 2010). Although classically considered to belong to the periglomerular cell subtype, dopaminergic INs form extensive interglomerular connections (Kosaka & Kosaka, 2008; Kiyokage et al., 2010), and are now classified primarily as short axon cells.
Several pieces of data indicate high plasticity of the OB dopaminergic neurons in response to manipulation of the olfactory pathway. Odour deprivation by naris occlusion, chemical or surgical OB deafferentation induces a drastic down-regulation of both TH mRNA and protein expression associated with a loss of detectable dopamine (Nadi et al., 1981; Kawano & Margolis, 1982; Baker et al., 1993; Philpot et al., 1998). Moreover, naris occlusion selectively reduces the number of adult-generated dopaminergic cells (Bovetti et al., 2009; Bastien-Dionne et al., 2010), which is recovered after naris reopening (Sawada et al., 2011).
Here, we asked whether, in physiological conditions, olfactory experience could influence dopaminergic cells in the OB. We used a paradigm of odour enrichment on adult mice, which has been previously shown to affect OB neurogenesis (Rochefort et al., 2002; Bovetti et al., 2009) and olfactory memory (Rochefort et al., 2002), and took advantage of TH–green fluorescent protein (GFP) transgenic mice (Sawamoto et al., 2001; Matsushita et al., 2002) to evaluate changes in the dopaminergic population.

Materials and methods

Animals

Experiments were performed on 8-week-old male C57BL/6J strain (Charles River, Calco, Italy) and TH–GFP mice. The generation of TH–GFP transgenic mice was as described previously (Sawamoto et al., 2001; Matsushita et al., 2002). TH–GFP mice were maintained as heterozygous by breeding heterozygous TH–GFP animals to C57BL/6J mice. All animals were housed under a 12-h light/dark cycle in an environmentally controlled room with food and water ad libitum. All experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC), Recommendation 18/06/2007, Dir. 2010/63/UE, and the Italian law for the care and use of experimental animals (DL116/92), and were approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin (DGSAF0011245-A-03/06/2013).

Odour enrichment

The C57BL/6J mice were held in standard laboratory cages in groups of five to seven and randomly assigned to the different experimental groups. The enriched (E) groups consisted of animals housed in an odour-exposure environment for 31 (E; $n = 5$) and 63 (E; $n = 7$) days. A further group of animals was maintained in enriched conditions for 42 days before returning to the standard housing condition for an additional 21 days [enriched followed by standard (E-S); $n = 7$]. Transgenic TH–GFP mice reared for 63 days in enriched environment (E; $n = 8$) or control conditions [standard (S); $n = 7$] were also added to this study. Odour-enriched mice were daily exposed for 24 h to different aromatic fragrances that were placed in a tea ball hanging from the acrylic filtering cover of standard breeding cages as previously described (Rochefort et al., 2002; Bovetti et al., 2009). Control mice ($n = 5$ for 31-day protocol; $n = 7$ for 63-day protocol) were reared under the same conditions except that the tea ball was left empty.

5-Bromo-2-deoxyuridine injections

To label newborn cells, adult TH–GFP mice received four intraperitoneal injections (4 h apart) of 5-bromo-2-deoxyuridine (BrdU) (50 mg/kg in 0.1 M Tris, pH 7.4; Sigma-Aldrich) on day 21 after the beginning of the odour enrichment. Following injection, mice were allowed to survive for a further 42 days in enriched or standard conditions depending on the experimental group.

Tissue preparation and sectioning

Mice were deeply anaesthetized by an intraperitoneal injection of a mixture of ketamine (Ketavet; Gellini) and xylazine (Rompun; Bayer) and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed from the skull, post-fixed for 4–6 h in the same solution, cryoprotected in a 30% sucrose solution in 0.1 M phosphate buffer (pH 7.4), frozen and cryostat sectioned (Leica Microsystems). Free-floating coronal serial sections (25 μm thick) containing the OB were collected in multiwell dishes. Sections were stored at -20 °C in antifreeze solution until use.

Immunofluorescence

For BrdU immunostaining, sections were treated with 2 N HCl for 35 min at 37 °C and neutralized with borate buffer, pH 8.5. Sections were incubated overnight at 4 °C in primary antibodies diluted in 0.01 M phosphate-buffered saline, pH 7.4, 0.5% Triton X-100, and 1% normal serum of the same species as the secondary antiserum. The primary antibodies used were: anti-BrdU (1 : 5000, rat; AbD Serotec), anti-calbindin (CB) D-28K (1 : 3000, mouse; Swant), anticalretinin (CR) (1 : 8000, mouse; Swant), anti-TH (1 : 2000, rabbit; Institut Jacques Boy, Reims, France), and anti-GFP (1 : 1000, chicken, Aves Lab.) (Table 1). For double labelling with BrdU, sections were first incubated overnight at 4 °C in anti-CB, anti-CR or anti-TH primary antibodies and the appropriate serum, and then for 1 h at room temperature (20–22 °C) in secondary antibodies. Sections were then processed for BrdU detection following the protocol described above. The secondary antibodies used were: anti-mouse, anti-rat and anti-rabbit Cy3-conjugated (1 : 800; Jackson ImmunoResearch), anti-mouse and anti-rabbit AlexaFluor 647-conjugated (1 : 600; Jackson ImmunoResearch), anti-chicken AlexaFluor 488-conjugated (1 : 400; Jackson ImmunoResearch), and anti-rabbit biotinylated (1 : 250; Vector Labs) followed by avidin–Fluorescein isothiocyanate (FITC) incubation (1 : 400; Vector Labs). Sections were mounted on gelatine-coated slides, air dried, and cover-slipped in polyvinyl alcohol with 1,4-diazabicyclo-octane as an antifading agent.

Microscopy and quantifications

All cell counts were conducted blind with regards to the experimental group. Cell counting and image analysis were performed on either a Nikon microscope coupled with a computer-assisted image analysis system (Neurolucida software, MicroBrightField) or a confocal microscope (TCS SP5, Leica).
To estimate the volume of each OB layer, camera lucida drawings of sections (six to eight sections per animal, 300-lm intersection intervals) through the entire OB were made from low-magnification photographs. The boundaries between layers were estimated from changes in cell density in sections stained with the nuclear dye 4 ' ,6-diamidino-2-phenylindole. The area of each section and layer in the traces was automatically calculated by Neurolucida software and the total volume of the OB and layers was estimated by applying the Cavalieri method (Prakash et al., 1994). The densities of positive cells were calculated by applying a random sampling method using a virtual counting grid. Three sections (positioned, respectively, at anterior, medial and posterior levels) per animal were

considered for cell counts for each marker. Cells were counted through the thickness of the slice in one pre-selected square by sequential translation of the counting grid until the entire layer of interest was covered. The cell density (D) was then calculated using the formula $D = (N * 4)/A*10^6$, where N is the number of positive cells counted using the grid and A is the GL area (μm 2), and expressed as the number of positive cells per mm^2.
Confocal image z-stacks were captured through the thickness of the slice at 1-lm optical steps and used for double-labelled cell counts. To establish the percentage of newly generated cells that were double-labelled for the different markers, serial sections from control and enriched mice were examined systematically through the OB (three sections positioned, respectively, at anterior, medial and posterior levels). All BrdU-positive (+) cells per slice were assessed to calculate the percentage of double-labelled cells for CB, CR and TH in the GL. To estimate the number of newly generated cells among the GFP+, TH+, CB+ or CR+ cell populations, we acquired four z-stacks (a dorsal, lateral, ventral and medial field per slice; three sections per animal positioned, respectively, at anterior, medial and posterior levels). For each animal, the fraction of newly generated cells among the marker-positive population was calculated by using manual cell counting on ImageJ software. The percentage of double-positive cells was calculated by counting the number of double-labelled cells among the totality of cells positive for the selected marker in the GL. Confocal pictures and reslicing were assembled into panels using the CorelDraw 11 software.

Statistical analysis

All data were firstly tested for fitting to the normal distribution (Kolmogorov–Smirnov's test) and, when normally distributed, statistical comparisons were conducted by two-tailed Student's t-test or one-way ANOVA followed by Tukey post-hoc comparison (alpha level = 0.05), where appropriate. Significance was established at $P < 0.05$. Cell counts and volumes are presented as mean ± SEM and are derived from at least three different animals, analysing at least three OB sections (at anterior, medial and posterior levels) for each animal. All statistical analyses were performed on either IBM SPSS Statistics 20 (Chicago, IL, USA) or Microsoft Excel (Office).

Results

Long-term and persistent odour enrichment increases tyrosine hydroxylase-immunopositive cells in the olfactory bulb glomerular layer

We investigated the effect of sensory enrichment on the dopaminergic cell population in the adult OB, following a previously established paradigm of odour enrichment (Fig. 1A) (Rochefort et al., 2002; Bovetti et al., 2009). Quantification of dopaminergic cells was carried out on OB tissue sections following immunolabelling for TH (Fig. 1B and E). Continuous exposure to the odour-enriched environment for 4 weeks did not result in changes in the TH+ cell density in the GL, the main site of dopaminergic cell integration in the OB (Fig. 1E) [Student's t-test, $P = 0.458$; S, $n = 5$ mice; E, $n = 4$ mice]. Interestingly, olfactory enrichment that lasted for 9 weeks induced a 21.80% increase in the density of TH+ neurons in the GL, whereas mice that were left to survive for 9 weeks after the beginning of the enrichment period but were returned to standard conditions for the last 3 weeks before analysis (E-S) showed no increment in TH+ cells compared with controls (Fig. 1E) (one-way ANOVA , $F_{2,9} = 34.556$, $P = 0.00006$; Tukey post-hoc: S vs. E, $P = 0.0001$; S vs. E-S, $P = 0.992$; E vs. E-S, $P = 0.0001$; $n = 4$ mice for each condition). Analysis of the GL volume among the different experimental groups (at 4 weeks: GL volume, 2.220 ± 0.054 mm^3 in S and 2.199 ± 0.076 mm^3 in E; at 9 weeks: GL volume, 2.078 ± 0.076 mm 3 in S, 2.184 ± 0.083 mm 3 in E and 2.130 ± 0.139 in E-S; $n = 3$ mice for each experimental group) revealed no differences (one-way ANOVA , $F_{4,10} = 0.408$, $P = 0.799$). We then analysed two other GL IN subtypes, namely CR+ and CB+ cells (Fig. 1C and D), which account for ~ 28 and ~ 10% of all GL neurons, respectively

(Parrish-Aungst et al., 2007). We did not find differences in cell densities of these populations following 9 weeks of continuous olfactory enrichment (Fig. 1F and G) (CR: Student's t-test, P = 0.165; S, n = 5 mice, E, n = 4 mice; CB: Student's t-test, P = 0.624; S and E, n = 3 mice).
On the whole, these data indicate that 9 weeks of odour-enriched conditions promote a selective increase in the population of TH+ cells in the OB GL, without altering CR+ and CB+ cell subtypes.

Prolonged olfactory enrichment in tyrosine hydroxylase–green fluorescent protein mice induces a parallel increase in tyrosine hydroxylase-positive and tyrosine hydroxylase-negative dopaminergic neurons in the olfactory bulb glomerular layer

It has been previously demonstrated that, in the OB, in addition to dopaminergic neurons expressing TH protein, there are also cells belonging to the dopaminergic lineage in which the transcription of the TH gene occurs in the absence of significant translational activity (Baker et al., 2001). Although the function of these elements is still largely unknown, they show electrophysiological properties related to immature dopaminergic neurons (Pignatelli et al., 2009). To investigate the effect of prolonged olfactory enrichment on this population, we took advantage of a transgenic mouse line expressing GFP under the TH promoter (Sawamoto et al., 2001; Matsushita et al., 2002). We first established the level of co-expression between TH protein and the TH–GFP transgene in the OB GL of adult animals (Fig. 2). Double immunostaining revealed that, whereas the large majority of TH+ cells were TH–GFP+ (n = 609 cells from four mice), only a fraction of the TH–GFP+ cells in this layer expressed TH protein (n = 936 cells from four mice), with nearly 40% of TH–GFP+ cells being TH-negative (Fig. 2A-D), in line with previous reports (Saino-Saito et al., 2004; Sawada et al., 2011).

Table 1. List and details of the primary antibodies used for immunofluorescence

	Supplier and cat. no.	Species of origin	Immunogen	Purification	Controls
Anti-BrdU	AbD Serotec, Bio-Rad Laboratories, Inc. (no. OBT0030CX)	Rat, monoclonal	Not reported	Not reported	Immunohistology and immunofluorescence on mouse brain tissue sections; flow cytometry analysis (view datasheet)
Anti-CB	Swant, Bellinzona, Switzerland (no. 300)	Mouse, monoclonal	CB D-28K purified from chicken gut	Not reported	Immunoblot of brain extracts; immunohistochemistry on CB D-28K knock-out mice (view datasheet)
Anti-CR	Swant (no. 6B3)	Mouse, monoclonal	Recombinant human CR-22k	Not reported	Immunoblot of brain extracts; immunohistochemistry on CR knock-out mice (view datasheet)
Anti-GFP	AvesLabs Inc., Tigard, OR, USA (no. GFP-1020)	Chicken, polyclonal GFP	Purified recombinant	Not reported	Western blot; immunohistochemistry using transgenic mice expressing the GFP gene product (view datasheet)
Anti-TH	Institut de Biotechnologie Jacques Boy, Reims, France (no. 208020234)	Rabbit, polyclonal	Pure TH isolated from pheochromocytoma	Not reported	Ouchterlony double immunodiffusion test; western blot (manufacturer's technical information)

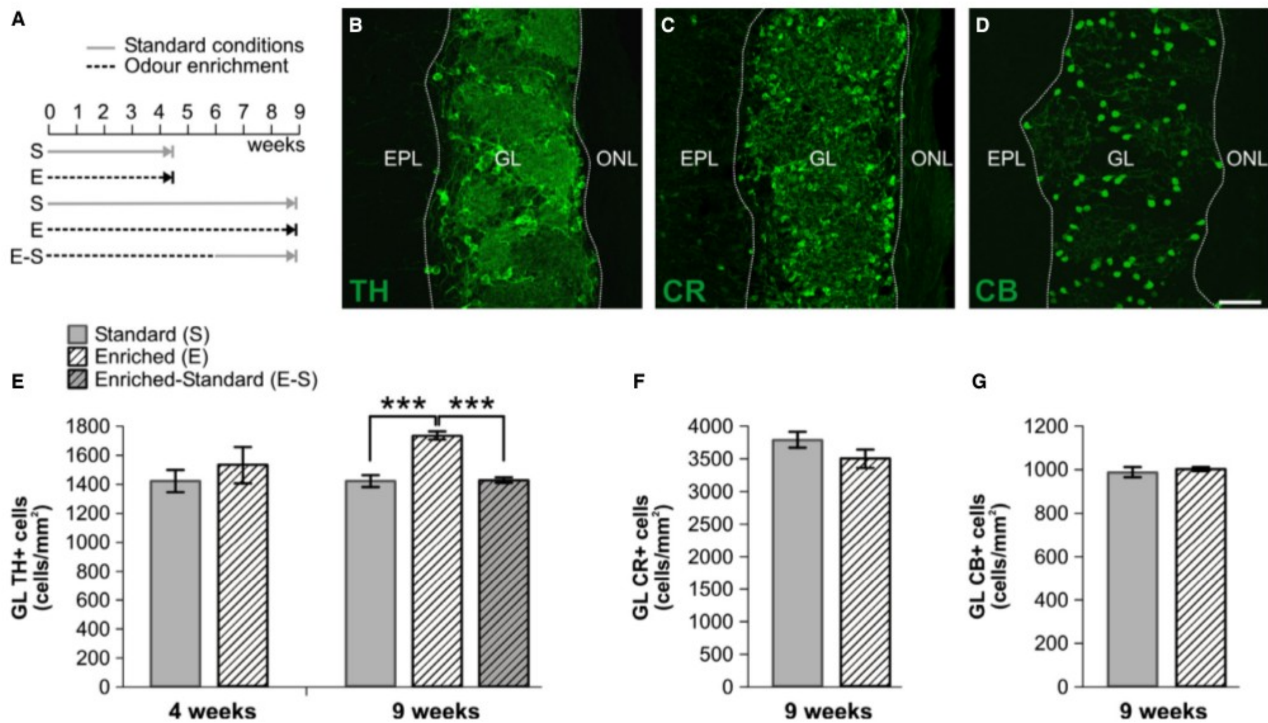


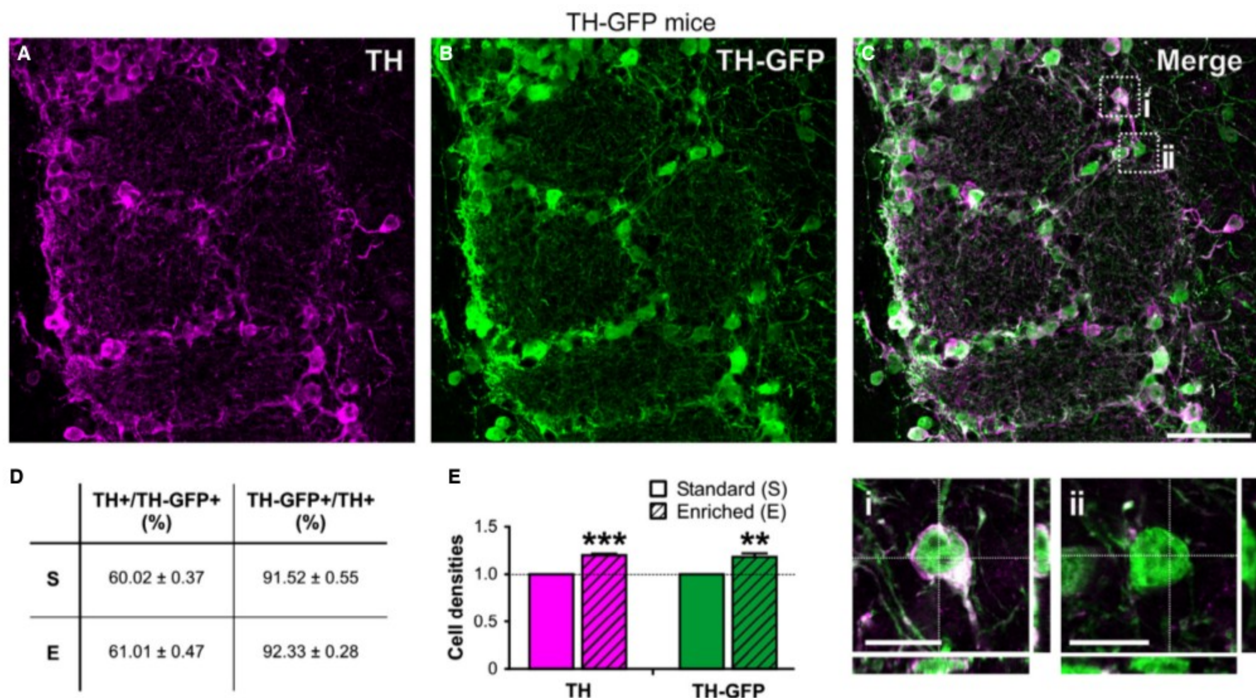
Fig. 1. Selective increase of TH+ cells in the adult OB GL under prolonged odour enrichment. (A) Experimental protocol. Representative images of the OB GL stained in green for TH (B), CR (C) or CB (D). (E) Density of TH-expressing cells in the OB GL of mice maintained in standard (S, grey), enriched (E, striped) or enriched followed by standard (E-S, striped dark grey) conditions at different survival times. Density of CR-expressing (F) and CB-expressing (G) cells in the OB GL in standard (S, grey) and enriched (E, striped) conditions at 9 weeks survival time. Data are presented as mean number of marker-positive cells/mm ^{2} . Error bars indicate SEM. Scale bar in D, 50 μm, also refers to B and C. EPL, external plexiform layer; ONL, olfactory nerve layer. Tukey post-hoc: ***P < 0.001.

As observed for naive mice, prolonged (9 weeks) olfactory enrichment on the TH–GFP transgenic line induced a statistically significant 20.19% increase of the TH+ population in the OB GL (Fig. 2E) (Student’s t-test, P = 0.000294; S: 1201.37 ± 45.39 mm ^{2} , n = 4 mice, E: 1444.00 ± 19.98 mm ^{2} , n = 7 mice). Interestingly, a similar increment was found for TH–GFP-expressing cells (Fig. 2E) (18.33% increase; Student’s t-test, P = 0.007488; S: 1486.89 ± 33.48 mm ^{2} , n = 4 mice, E: 1759.43 ± 55.86 mm ^{2} , n = 7 mice). Notably, the percentage of co-expression between TH+ and TH- GFP+ cells remained unchanged (Fig. 2D) (TH–GFP+/TH+: Student’s t-test, P = 0.175; n = 609 cells from four S mice; n = 1243 cells from seven E mice; TH+/TH–GFP+: Student’s t-test, P = 0.184; n = 936 cells from four S mice; n = 2018 cells from seven E mice), indicating that odour enrichment increased the whole dopaminergic population, including the TH-negative, putative immature dopaminergic cells. No changes were observed in the GL volume among the experimental groups (GL volumes: S, 2.008 ± 0.069 mm ^{3} , n = 3 mice; E, 2.026 ± 0.046 mm ^{3} , n = 3 mice; Student’s t-test, P = 0.838).

Adult neurogenesis underlies the tyrosine hydroxylase-positive cell increase following olfactory enrichment

We have previously demonstrated increased neurogenesis in the GL of adult mice following olfactory enrichment (Bovetti et al., 2009). To investigate whether enhanced neurogenesis could account for the enlargement of the dopaminergic population, we injected TH–GFP mice with BrdU at 3 weeks after the beginning of the enrichment protocol, and continued the enrichment for 6 weeks (Fig. 3A). In this way, we were able to label adult-generated cells that were born in an enriched environment and analyse them at a fully mature stage, at the end of an enrichment paradigm that lasted 9 weeks in total. In line with our previous study (Bovetti et al., 2009), we

found that the density of BrdU+ cells in the GL was increased by 35.56% in enriched vs. standard mice (Fig. 3B) (Student's t-test, P = 0.012; S and E, n = 4 mice). Next, the percentage of BrdU+ neurons expressing TH, TH–GFP, CR or CB in enriched conditions was compared with that in standard mice (Fig. 3C–N). Odour enrichment significantly increased the number of TH+/BrdU+ and TH–GFP+/BrdU+ double-labelled cells in the GL (Fig. 3C and D, and F and G) (TH+/BrdU+: Student's t-test, P = 0.035; n = 943 cells from seven S mice; n = 947 cells from eight E mice; TH–GFP+/BrdU+: Student's t-test, P = 0.012; n = 155 cells from four S mice, n = 202 cells from four E mice). However, no changes were observed in the percentage of double-labelled CR+/BrdU+ or CB+/BrdU+ GL INs (Fig. 3I and J; CR+/BrdU+: Student's t-test, P = 0.488, n = 475 cells from four S mice, n = 578 cells from four E mice; Fig. 3L and M; CB+/BrdU+:



	TH+/TH-GFP+ (%)	TH-GFP+/TH+ (%)
S	60.02 ± 0.37	91.52 ± 0.55
E	61.01 ± 0.47	92.33 ± 0.28

Fig. 2. Prolonged odour enrichment on TH–GFP mice. (A-C) Representative confocal images of the OB GL stained for TH (A, magenta) and TH–GFP (B, green). A merged image is shown in C. Insets in C are displayed at higher magnification with reslices in Ci and Cii, and show a cell double-labelled for GFP and TH (Ci) and a cell positive for GFP and negative for TH (Cii). (D) Percentage of double labelling for TH and TH–GFP in standard (S) and enriched (E) mice. (E) Normalized cell densities of TH+ (pink) and TH–GFP+ (green) cells in 9-week enriched mice (E, striped) compared with controls (S, not striped). Error bars indicate SEM. Student's t-test: **P < 0.01; ***P < 0.001.

Student's t-test, P = 0.560, n = 211 cells from four S mice, n = 252 cells from four E mice). Strikingly, the percentage of BrdU+ cells among the TH+ and TH–GFP+ populations doubled in enriched animals (Fig. 3E and H) (BrdU+/TH+: Student's t-test, P = 0.012, n = 451 cells from three S mice, n = 615 cells from four E mice; BrdU+/TH–GFP+: Student's t-test, P = 0.0012, n = 389 cells from three S mice, n = 462 cells from three E mice), further indicating a net addition of new dopaminergic cells under enrichment conditions. Moreover, no differences were found in terms of the contribution of BrdU+ newly generated cells in enriched vs. control mice in the case of the CR+ population (Fig. 3K) (Student's t-test, P = 0.373, n = 1435 cells from four S mice, n = 1353 cells from four E mice) and CB+ population (Fig. 3N) (Student's t-test, P = 0.678, n = 486 cells from four S mice, n = 448 cells from four E mice).

Discussion

A large number of studies have demonstrated that afferent synaptic activity in the olfactory sensory neurons controls TH expression in the OB (for review see Cave & Baker, 2009), modulating the dopaminergic inhibitory glomerular network. Specifically, odour deprivation reduces TH expression and decreases OB dopamine content, whereas recovery of the olfactory input after reversible deafferentation results in restoration of both TH and dopamine (Baker et al., 1983). In addition, more recent studies have shown that naris occlusion also impacts on adult neurogenesis by selectively decreasing the number of newborn dopaminergic neurons integrated in the GL (Bovetti et al., 2009; Bastien-Dionne et al., 2010), whereas reopening of the nostril results in increased survival of newborn dopaminergic cells (Sawada et al., 2011). In this study, we monitored the olfactory dopaminergic population in mice reared in an odour-enriched environment, a condition that has been previously demonstrated to impact on behavioural olfactory responses (Rochefort et al., 2002) and to induce increased neuronal activity, compared with mice in standard housing (Woo et al., 2007).

Importantly, the paradigm used in this study does not imply perturbation of the olfactory system, like that occurring in olfactory deprivation/recovery experiments, providing a more physiological frame to test the effect of sensory experience on the dopaminergic population.

A main finding of the present study was that prolonged and persistent odour-enriched exposure results in increased TH+ dopaminergic cells in the OB GL. How is this expansion achieved? One possibility is that, in the presence of a sustained increased olfactory activity, resident OB cells expressing TH mRNA but lacking TH protein (Baker et al., 2001), and showing electrophysiological properties typical of immature dopaminergic neurons (Pignatelli et al., 2009), could be recruited and induced to fully differentiate, translating TH transcript into functional TH protein. To investigate this possibility, we took advantage of a transgenic mouse line expressing GFP under the TH promoter (Sawamoto et al., 2001; Matsushita et al., 2002), in which nearly 40% of the TH–GFP+ elements in the OB GL do not express TH protein in standard conditions. Following odour enrichment exposure on these mice, we found an expansion of the whole TH–GFP+ cell population, with no change in the ratio between TH+ and TH-negative cells among the TH–GFP+ elements, indicating no effects on the translational control of TH protein in putative immature INs. Instead, the increase in dopaminergic cells could be dependent on enhanced neurogenesis. It is well established that OB INs, including dopaminergic cells, are continuously generated throughout adulthood (Kohwi et al., 2005, 2007; Mizrahi et al., 2006; Bagley et al., 2007; Bovetti et al., 2007; De Marchis et al., 2007; Adam & Mizrahi, 2011; Sawada et al., 2011). Moreover, we have previously shown that odour enrichment enhances the survival of adult-generated cells in the OB GL (Bovetti et al., 2009). Here, we showed for the first time that this effect selectively impacts on the dopaminergic population. Indeed, following prolonged odour enrichment we found increased TH+ and TH–GFP+ cells among the 6-week-old BrdU+ cells generated during the period of sensory enrichment. More importantly, we showed a twofold ncrease in the percentage of newborn BrdU+ cells among the TH+ and TH–GFP+ cell populations in enriched conditions. In our previous work (Bovetti et al., 2009), enhanced neurogenesis did not correspond to an increase of adult-generated TH+ cells under the odour-enriched condition. However, our former analysis was performed on 3-week-old BrdU+ cells (Bovetti et al., 2009), instead of 6-week-old BrdU+ cells (this study). It is noteworthy that, in the adult OB GL, the percentage of newborn BrdU+ cells double labelled for TH slightly increases during the first 4 weeks after BrdU injection, to rise drastically afterwards reaching a peak at about 6 weeks (Kohwi et al., 2007), indicating a slow maturation profile for this IN subpopulation. Thus, it is likely that in

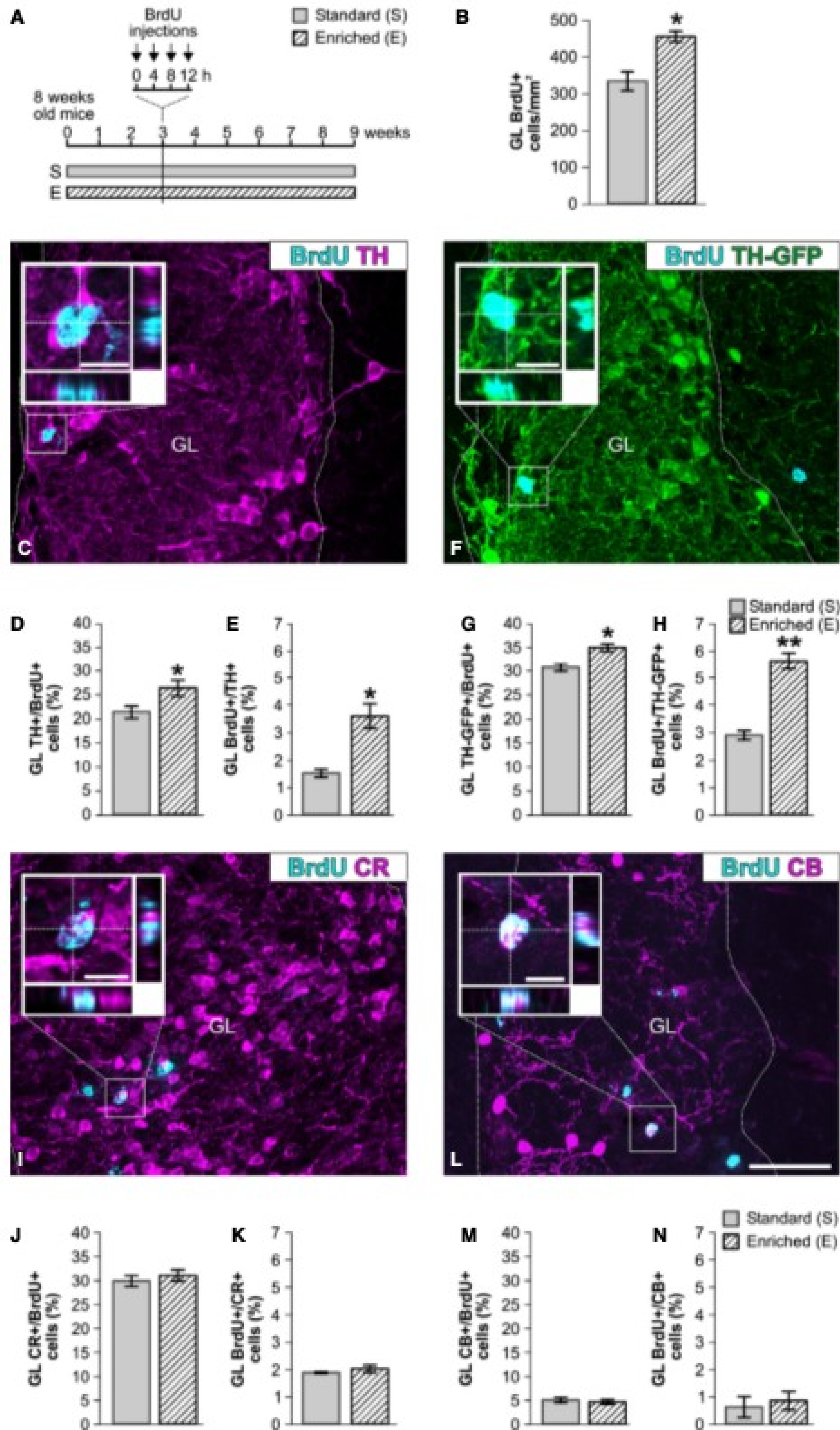


Fig. 3. The odour-enriched environment selectively enhanced adult-born dopaminergic INs in the OB GL. (A) Experimental protocol. (B) Mean number of BrdU+ cells/mm 2 in the GL of standard (S, grey) and enriched (E, striped) mice. Representative confocal images of the OB GL double-stained in cyan for BrdU, in magenta for TH (C), CR (I) or CB (L), or in green for TH–GFP (F). Insets show higher magnifications of resliced double-labelled BrdU+/marker-positive cells. (D, G, J and M) Quantification of adult newly generated cells (BrdU+) expressing TH (D), TH–GFP (G), CR (J) or CB (M) among the total number of counted BrdU+ cells in the GL of enriched (E, striped) and control (S, grey) mice. Quantification of the fraction of BrdU+ cells among the total number of TH+ (E), TH–GFP+ (H), CR+ (K) or CB+ (N) cells counted in the GL of enriched (E, striped) and control (S, grey) mice. Scale bar in L, 50 μm, also refers to C, F and I. Scale bars in inserts, 10 lm. Error bars indicate SEM. Student's t-test: *P < 0.05; **P < 0.01.

our previous study we missed the effect of sensory enrichment on the dopaminergic population because we were focussed on an early stage of cell maturation (3 weeks), when most BrdU+ cells specified to become dopaminergic do not yet express TH protein. This also supports exposure to odour enrichment not influencing the maturation of dopaminergic cells.
In the current study, as well as in our previous observations (Bovetti et al., 2009), we did not find any changes in the percentage of BrdU+ cells that were double labelled for CB+ or CR+ in enriched vs. standard mice. Moreover, no differences were observed following odour enrichment in the percentages of BrdU+ cells in the CB+ and CR+ glomerular IN subtypes, confirming that sensory enrichment does not imply a general increment of glomerular INs but selectively enhances integration of new dopaminergic cells. Accordingly, CB+ and CR+ populations in the GL of enriched mice showed density values equivalent to that found in standard mice. These observations, together with previous studies (Bastien-Dionne et al., 2010; Sawada et al., 2011), strongly indicate dopaminergic cells as a key cellular substrate in the neuronal plasticity mechanisms underlying the adaptation of the olfactory system to changes in sensory experience. None to low sensory activity drastically tunes down the inhibitory dopaminergic network, whereas sensory enrichment enhances it. The effect of the sensory input on dopaminergic cell plasticity appears to be twofold, regulating TH expression and consequently dopamine content in residing dopaminergic cells on one side, and acting on the survival and integration of new dopaminergic elements on the other side. Our data clearly show that sensory enrichment acts by promoting integration of new elements in the circuits; however, we cannot exclude that effects on the pre-existing population of dopaminergic neurons could also occur in parallel, i.e. by enhancing their survival and/or the level of TH expression in TH+ cells.
It is likely that the effects on cell survival and TH expression are mediated by specific cellular/molecular mechanisms. Accordingly, we have recently shown that the transcription factor Chicken Ovalbumin Upstream Promoter Transcription Factor I, which is selectively expressed in mature dopaminergic cells in the GL, regulates activity-dependent TH expression, having no effects on cell survival (Bovetti et al., 2013). Further studies should investigate the mechanisms underlying the newborn dopaminergic cell increase by sensory experience. Moreover, how changes in the dopaminergic inhibitory network impact on odour processing remains a major question to be addressed.

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Abbreviations

BrdU, 5-bromo-2' -deoxyuridine; CB, calbindin; CR, calretinin; E, enriched; E-S, enriched followed by standard; GFP, green fluorescent protein; GL, glomerular layer; IN, interneuron; OB, olfactory bulb; S, standard; TH, tyrosine hydroxylase.

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