RESEARCH ARTICLE

COUP-TFI controls activity-dependent tyrosine hydroxylase expression in adult dopaminergic olfactory bulb interneurons

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ABSTRACT

COUP-TFI is an orphan nuclear receptor acting as a strong transcriptional regulator in different aspects of forebrain embryonic development. In this study, we investigated COUP-TFI expression and function in the mouse olfactory bulb (OB), a highly plastic telencephalic region in which continuous integration of newly generated inhibitory interneurons occurs throughout life. OB interneurons belong to different populations that originate from distinct progenitor lineages. Here, we show that COUP-TFI is highly expressed in tyrosine hydroxylase (TH)-positive dopaminergic interneurons in the adult OB glomerular layer (GL). We found that odour deprivation, which is known to downregulate TH expression in the OB, also downregulates COUP-TFI in dopaminergic cells, indicating a possible correlation between TH- and COUP-TFI-activity-dependent action. Moreover, we demonstrate that conditional inactivation of COUP-TFI in the EMX1 lineage results in a significant reduction of both TH and ZIF268 expression in the GL. Finally, lentiviral vector-mediated COUP-TFI deletion in adult-generated interneurons confirmed that COUP-TFI acts cell-autonomously in the control of TH and ZIF268 expression. These data indicate that COUP-TFI regulates TH expression in OB cells through an activity-dependent mechanism involving ZIF268 induction and strongly argue for a maintenance rather than establishment function of COUP-TFI in dopaminergic commitment. Our study reveals a previously unknown role for COUP-TFI in the adult brain as a key regulator in the control of sensory-dependent plasticity in olfactory dopaminergic neurons.

KEY WORDS: Juxtaglomerular cells, Tyrosine hydroxylase (TH), EMX1 lineage, Sensory deprivation, ZIF268, Mouse

INTRODUCTION

In the olfactory bulb (OB), a rich and heterogeneous population of glomerular layer (GL) interneurons participates in the first steps of olfactory information processing by modulating the activity of the OB output neurons, the mitral/tufted cells. Most juxtaglomerular cells are GABAergic and can be classified into different subclasses based on the expression of specific neurochemical markers and the connection networks they establish within the GL (Parrish-Aungst et al., 2007; Kosaka and Kosaka, 2007; Kiyokage et al., 2010). For example, calbindin-positive (CB+) and calretinin-positive (CR+) cells are activated by the inputs from the mitral/tufted dendrodendritic synapses and are involved in local intraglomerular circuits. Cells expressing tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine (DA) synthesis, in addition to inputs from mitral/tufted cells, also receive direct synaptic inputs from the olfactory nerve terminals and can be involved in either intraglomerular or interglomerular (long-range) connections (Kiyokage et al., 2010). Although TH+ DAergic interneurons represent only the 10-15% of juxtaglomerular cells, they correspond to a population of particular interest in the OB. They play a key role in olfactory perception, discrimination and learning (Cave and Baker, 2009). Interestingly, TH expression and consequently DA release in GL interneurons depend on afferent synaptic activity and are strongly reduced following olfactory deprivation. This activity-dependent regulation of TH has been related to a possible role of DA in the modulation of odorant information processing in response to either high or low levels of afferent odour-induced synaptic activity (Cave and Baker, 2009).

As for other OB interneurons, the generation of DAergic cells initiates during embryonic ages and continues throughout life from spatially restricted pools of stem/progenitor cells (Luskin, 1993; De Marchis et al., 2007; Young et al., 2007; Merkle et al., 2007; Batista-Brito et al., 2008). The earliest generated DAergic cells are locally derived from stem cells in the prospective OB (Vergaño-Vera et al., 2006). Starting from mid-embryonic development, DAergic cells largely originate from subventricular zone (SVZ) and rostral migratory stream (RMS) progenitors that mostly belong to the DLX5/6 lineages (derived from the subpallium) or EMX1 lineages (derived from the pallium) at different ratios depending on age (Hack et al., 2005; Kohwi et al., 2007; Young et al., 2007). Although several genes involved in the specification and differentiation of the OB DAergic neurons, including PAX6, DLX2 and ER81 (ETV1 – Mouse Genome Informatics), have been identified (Hack et al., 2005; Brill et al., 2008; de Chevigny et al., 2012; Saino-Saito et al., 2007; Cave et al., 2010), the complexity of the molecular genetic regulation of OB DAergic interneurons is far from being elucidated.

In this study, we analysed the function of the chicken ovalbumin upstream promoter transcription factor I (COUP-TFI; NR2F1 – Mouse Genome Informatics) in olfactory DAergic neurons. COUP-TFI, a member of the orphan nuclear receptor family, plays multiple roles in neuronal development (Park et al., 2003; Alfano et al., 2013). It is highly expressed in the developing telencephalon (Qu et al., 1994; Armentano et al., 2006), where it regulates the balance of cortical patterning between frontal/motor and sensory areas (Armentano et al., 2007), and is involved in the migration and

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specification of cortical GABAergic interneurons in the subpallium (Tripodi et al., 2004; Lodato et al., 2011). Here, we show that COUP-TFI is co-expressed with TH in the vast majority of DAergic neurons in the OB, in an activity-dependent manner. By means of genetic ablation of COUP-TFI function in EMX1 and DLX5/6 progenitor lineages, we provide evidence that in the adult OB, COUP-TFI acts selectively in EMX1-derived interneurons controlling the expression of TH and of the immediate early gene Zif268 [also known as Egr1 or NGFI-A (Knapska and Kaczmarek, 2004)]. Finally, by cell-autonomous manipulations in adult RMS progenitors, we demonstrate a COUP-TFI intrinsic function in the control of TH and ZIF268 expression in the adult OB.

RESULTS
COUP-TFI is expressed in mature DAergic olfactory interneurons
COUP-TFI expression in the adult OB was investigated by immunofluorescence (Fig. 1). Intense COUP-TFI nuclear immunolabelling was found in cells mostly localised in the OB GL and granule cell layer (GcL). In the GL most immunopositive nuclei showed dense labelling, whereas in the GcL COUP-TFI+ nuclei displayed a wide range of staining intensity, from strong to very low (Fig. 1A). To further characterise COUP-TFI+ positive cells in the OB, we performed double labelling for COUP-TFI and either NeuN (RBFOX3 – Mouse Genome Informatics) (Fig. 1B) or doublecortin (DCX; Fig. 1C). The neuron-specific nuclear protein NeuN is expressed by all mature granule cells in the GcL and by a subset of mature cells in the GL (Bagley et al., 2007), whereas DCX is expressed by all SVZ-derived neuroblasts and progressively downregulated as interneurons mature (Brown et al., 2003). The large majority of COUP-TFI+ cells was co-labelled with green fluorescent

![Fig. 1. COUP-TFI distribution in the adult and postnatal mouse OB.](image-url)

(A-D) Adult OB coronal sections stained in red for COUP-TFI and in green for NeuN (B), DCX (C) or GAD67-GFP (D) (GAD67 also known as GAD1 – Mouse Genome Informatics); insets in B and C show double-labelled cells in the GL; insets in D show confocal images of double-labelled cells in the GL and GcL at higher magnification. (E-I), Adult OB GL stained in red for COUP-TFI and in green for CR (E), CB (F), TH (G, inset: confocal image at higher magnification), GFP expressed under TH promoter (H) or PAX6 (I). (J-M) OB GL at different postnatal (P) ages (P0, J; P7, K; P14, L; P21, M) stained in red for COUP-TFI and in green for TH. The arrowheads indicate double-labelled cells. In I, the arrow indicates a PAX6 cell negative for COUP-TFI. (N-O) Quantification of TH+ cells (N) and COUP-TFI+ cells (O) at different postnatal ages. (P-Q) Quantification of COUP-TFI and TH cells double-labelled for TH and COUP-TFI, respectively. P0, n=4; P7, n=3; P14, n=3; P21, n=3; adult, n=6. Error bars indicate s.e.m. Tukey post-hoc; ***P<0.001; ns, not significant, P>0.05. Scale bars: 100 μm in D for D,A; 10 μm in insets in D,G; 50 μm in I for B,C,E-I; 50 μm in M for J-M; 50 μm in insets in B,C. EPL, external plexiform layer; GcL, granule cell layer; GL, glomerular layer.
TH+ population starting from the second postnatal week. A similar pattern was observed for COUP-TFI cells (one-way ANOVA, \(F_{(4,13)}=251.11, P<0.001\); Fig. 1O). Double labelling analysis showed that as for adult mice, also in newborn or young animals a substantial fraction (ranging from 70 to 80%) of the COUP-TFI+ population was double positive for TH (Fig. 1P). Interestingly, the percentage of TH+ cells expressing COUP-TFI in the OB changed with time. In the first postnatal week the large majority of TH+ cells was negative for COUP-TFI (Fig. 1Q). Conversely, 55% and 62% of TH+ cells expressed COUP-TFI at P14 and P21, respectively, which further increased to 80% in adult mice (one-way ANOVA, \(F_{(4,13)}=908.52, P<0.001\); Fig. 1Q), confirming that expression of COUP-TFI is associated with DAergic cells generated during late postnatal and adult life.

**Sensory input regulates COUP-TFI expression in olfactory DAergic cells**

It is well known that TH expression in DAergic cells depends on afferent synaptic activity and that odour deprivation causes TH downregulation (Baker et al., 1993). As COUP-TFI is associated with mature DAergic cells, we asked whether odour deprivation could also affect COUP-TFI expression in the GL. To address this issue, odour deprivation was performed by unilateral nasir closure and the total number of TH+ and COUP-TFI+ cells was estimated in control (Ctr) and deprived (Depr) mice 42 days after nasir closure (Bovetti et al., 2009). As expected, a strong decrease in the number of TH+ cells was observed in the OB ipsilateral to the closure (Ctr versus Depr: \(t\)-test \(P<0.001\); Fig. 2A-C). In parallel, we detected nearly 50% reduction of COUP-TFI+ neurons in the GL (Ctr versus Depr: \(t\)-test \(P<0.001\); Fig. 2A,B,D). Accordingly, the number of double TH+/COUP-TFI+ labelled cells was significantly decreased (71.48±0.87% in controls, \(n=1558\) cells; 21.32±1.06% in deprived, \(n=1131\) cells; \(t\)-test \(P<0.001\); Fig. 2D), indicating that COUP-TFI downregulation occurred jointly with TH downregulation. These data suggest possible implications of COUP-TFI function in the activity-dependent regulation of TH expression in DAergic cells.

**Genetic inactivation of COUP-TFI in the EMX1 lineage affects TH expression in adult-generated olfactory interneurons**

To investigate the role of COUP-TFI in the DAergic phenotype, we chose a conditional genetic approach. We obtained mice lacking COUP-TFI in either the DLX5/6 or the EMX1 lineage [i.e. two of the main lineages giving rise to OB interneurons (Kohwi et al., 2002; Young et al., 2007)] by crossing the COUP-TFI-floxed line (COUP-TFI\(fl/fl\)) (Armentano et al., 2007) to either the Dlx5/6-\(IRES\)-Cre (Stenman et al., 2003) or the Emx1-\(IRES\)-Cre (Gorski et al., 2002) transgenic lines. There was no apparent loss of COUP-TFI expression in the olfactory GL of COUP-TFI\(fl/fl\)Dlx5/6-Cre mice (one-way ANOVA, \(F_{(2,10)}=82.845, P<0.001\); \(fl/fl\) versus \(fl/fl\)Emx1-Cre, Tukey post-hoc \(P=0.469\); Fig. 3A,D,J). Similarly, no changes were detected in the density of TH+ cells in these animals (one-way ANOVA, \(F_{(2,8)}=32.009, P<0.001\); \(fl/fl\) versus \(fl/fl\)Emx1-Cre, Tukey post-hoc \(P=0.480\); Fig. 3B,E,K). On the contrary, in mice in which COUP-TFI had been deleted in the EMX1 lineage showed a drastic reduction of COUP-TFI+ cells in the GL (one-way ANOVA, \(F_{(2,10)}=82.845, P<0.001\); \(fl/fl\) versus \(fl/fl\)Emx1-Cre, Tukey post-hoc \(P<0.001\); Fig. 3A,G,J), indicating that COUP-TFI-expressing juxtaglomerular cells mainly originate from the EMX1 lineage rather than from DLX5/6-derived progenitors. However, no changes in the GL volume were observed (1.8±0.06 mm\(^3\) in \(fl/fl\) mice and 1.8±0.06 mm\(^3\) in \(fl/fl\)Emx1-Cre mice, \(t\)-test \(P=0.46\)).
Importantly, analysis of TH immunofluorescence in the OB of COUP-TFIfl/flEmx1-Cre mice showed a net reduction in the density of TH + cells (one-way ANOVA, F(2,8)=32.009, P<0.001; fl/fl versus fl/flEmx1-Cre, Tukey post-hoc P<0.001; Fig. 3B,H,K), which was lower than the one observed in COUP-TFI + cells (Fig. 3J,K). Accordingly, TH+ cells in COUP-TFIfl/flEmx1-Cre mice co-express COUP-TFI+ in a lower percentage compared with control mice (26.56±1.73% of TH + cells were COUP-TFI + in fl/flEmx1-Cre, n=496 cells; 80.81±0.36% of TH + cells were COUP-TFI + in fl/fl, n=738 cells; t-test P<0.001; Fig. 3C,I), indicating that COUP-TFI ablation affects TH expression only in a subset of DAergic cells.

We next analysed COUP-TFI and TH expression in young postnatal (P7) COUP-TFIfl/flEmx1-Cre mice and found no changes in the density of both COUP-TFI+ and TH+ cells compared to control mice (COUP-TFI+: 732±38 cells/mm² in fl/fl and 688±20 cells/mm² in fl/flEmx1-Cre, t-test P=0.43; TH+: 677±91 cells/mm² in fl/fl and 497±14 cells/mm² in fl/flEmx1-Cre, t-test P=0.19). This suggests that COUP-TFI is not involved in TH regulation of early-generated DAergic cells deriving from the EMX1 lineage.

We then asked whether absence of COUP-TFI function in the EMX1 lineage could also alter, besides TH, transcription factors, such as PAX6 and ER81, which are known to play a role in DAergic interneuron generation. No differences were found either in PAX6+ or ER81+ cell densities (PAX6+: t-test P=0.950; ER81+: t-test P=0.10; Fig. 4D,E) or in the percentages of BrdU+ cells double labelled for PAX6 or ER81 (PAX6+/BrdU+: 55.63±0.66% in fl/fl, n=104 cells; 56.40±3.37% in fl/flEmx1-Cre, n=99 cells; t-test P=0.83; ER81+/BrdU+: 54.77±0.28% in fl/fl, n=115 cells; 59.37±3.69% in fl/flEmx1-Cre, n=106 cells; t-test P=0.28). In parallel, analysis of CR+ and CB+ cells in the GL of COUP-TFIfl/flEmx1-Cre mice showed no effect on these juxtaglomerular populations (CB+: t-test P=0.487; Fig. 4F,G).

Together, these data indicate that loss of COUP-TFI expression in the EMX1 lineage selectively affects adult-generated DAergic cells, where it regulates TH expression, without altering their survival or expression of factors related to the DAergic lineage.
Impaired ZIF268 expression in COUP-TFIIfl/Emx1-Cre mice
A possible explanation of the TH phenotype observed in the absence of COUP-TFI in the EMX1 lineage is that COUP-TFI inactivation could affect TH expression in OB DAergic cells through an activity-dependent mechanism. To address this hypothesis, we examined the level of expression of the product of the immediate early gene Zif268 in the GL of COUP-TFIIfl/Emx1-Cre and control mice. ZIF268 has been suggested to mediate TH activity-dependent expression in a subset of OB DAergic neurons (Akiba et al., 2009) and, importantly, previous studies have identified ZIF268 as a possible target for COUP-TFI (Durr et al., 2007) and, importantly, previous studies have identified ZIF268 as a possible target for COUP-TFI through direct interactions with SP1 (Pipáon et al., 1999). Both COUP-TFIfl/flEmx1-Cre and control mice were tested in an experimental paradigm (modified from Vincis et al., 2012; Magavi et al., 2005) (Fig. 5A) in which animals were maintained either in conditions of low odour exposure (basal) or acutely stimulated by an odour mix (stim). In COUP-TFIfl/flEmx1-Cre mice, we detected a lower density of juxtaglomerular cells expressing ZIF268 compared with control mice in basal conditions (two-way ANOVA, F(3,9)=124.41, P<0.01; basal fl/+/versus basal fl/flEmx1-Cre, Tukey post-hoc P<0.001; Fig. 5B-D). Although COUP-TFIfl/flEmx1-Cre mice responded to the odour mix stimulation with an increase in the density of ZIF268+ cells (basal fl/flEmx1-Cre versus stim fl/flEmx1-Cre, Tukey post-hoc P<0.01; Fig. 5B,E,F), the average value reached in mutants was considerably lower compared with that observed in control mice (stim fl/fl versus stim fl/flEmx1-Cre, Tukey post-hoc P<0.001; Fig. 5B), indicating an impairment in juxtaglomerular cell activation both in basal conditions and upon acute odour stimulation.

COUP-TFI controls TH expression in mature DAergic cells through a cell-autonomous activity-dependent mechanism
To understand further the mechanisms at the basis of the activity-dependent control of TH by COUP-TFI and assess whether the effects of COUP-TFI ablation observed in COUP-TFIfl/Emx1-Cre were cell-autonomous, we used a lentiviral vector approach. Based on previous reports indicating that RMS precursors give rise to a significantly larger population of GL interneurons compared with SVZ precursors (Hack et al., 2005; Ihrie and Alvarez-Buylla, 2011), a lenti-CRE-iGFP virus was injected into the RMS of the left hemisphere in adult COUP-TFIIfl/fl mice, in order to inactivate COUP-TFI in a subset of juxtaglomerular cell progenitors/precursors (Fig. 6A-C). In parallel, the right hemispheres of the same mice were injected with control lenti-iGFP virus (Fig. 6A). Mice were then left to survive for 30 or 60 days post-injection (dpi) before analysis (Fig. 6B). At both ages, GFP+ (Fig. 6F) and CRE+/GFP+ (Fig. 6J,K,N,O) cells were found in the GL, in the right and left hemispheres, respectively, of injected COUP-TFIfl/fl mice. In lenti-iGFP injected mice, the percentage of GFP+ cells double labelled for COUP-TFI ranged from nearly 24% to 36% at 30 and 60 dpi, respectively (30 dpi, n=289 infected cells; 60 dpi, n=366 infected cells; Fig. 6D-H). Importantly, the quantification of COUP-TFI+ cells in lenti-CRE-iGFP injected hemispheres showed no COUP-TFI immunoreactivity in CRE+/GFP+ cells (30 dpi, n=183 infected cells; 60 dpi, n=345 infected cells; Fig. 6D,L-L), indicating high efficiency of CRE-recombinase activity in infected cells. As control, we also injected lenti-CRE-iGFP viruses in wild-type mice and found CRE+/GFP+ cells positive for COUP-TFI (30 dpi: 35.28±4.08%, n=159 injected cells; 60 dpi: 44.2±0.8%, n=246 injected cells), confirming the specificity of CRE activity in COUP-TFIfl/fl mice. In lenti-iGFP injected COUP-TFIfl/fl mice, the percentage of double-labelled TH+/GFP+ cells was 21% and 36% at 30 and 60 dpi, respectively (30 dpi n=289 cells; 60 dpi n=366 cells; Fig. 6D-H,M-P), whereas the percentage of triple-labelled TH+/COUP-TFI+/GFP+ cells was slightly lower (30 dpi: 15.98±3.44%, n=289 infected cells; 60 dpi: 25.63±0.99%, n=366 infected cells; Fig. 6D), in line with a large but incomplete co-expression between TH and COUP-TFI in juxtaglomerular cells (Fig. 1P,Q). As we hypothesised a cell-autonomous function for COUP-TFI on TH, we expected a decrease in TH expression in the CRE+/GFP+ population of lenti-CRE-iGFP injected hemispheres. Accordingly, we observed a net reduction of TH expression in CRE+/GFP+ cells at 60 dpi (60 dpi lenti-iGFP, n=366 cells versus lenti-CRE-iGFP, n=345 cells, t-test P<0.001; Fig. 6D), but no difference in the percentage of TH+ cells at 30 dpi (30 dpi lenti-iGFP, n=289 cells versus lenti-CRE-iGFP, n=156 cells, t-test P=0.379; Fig. 6D). No differences were found in CR expression between lenti-iGFP and lenti-CRE-iGFP injected hemispheres (60 dpi, lenti-iGFP: 38.1±1.1%, n=208 infected cells; lenti-CRE-iGFP: 35.2±3.5%, n=177 infected cells; t-test
CRE+/GFP+ cells (Fig. 7) and found a statistically significant decrease of CRE.

The majority of PAX6+ and TH+ cells in the GL are found to be positive; they largely belong to the DAergic lineage. Notably, the large number of BrdU+ cells that were double positive for TH, in line with the observed decrease of TH+ cells over the whole GL interneuron population. Moreover, no differences were found in the density of PAX6+/BrdU+ and R81+/BrdU+ cells, in the GL of COUP-TFIfl/flEmx1-Cre mice. This strongly indicates that absence of COUP-TFI in EMX1 lineage only marginally contributes to COUP-TFI-expressing cells in the adult OB.

DISCUSSION

In the present study, we demonstrate a novel key function of the nuclear receptor COUP-TFI in the maintenance of the DAergic phenotype in the adult OB. COUP-TFI has been thoroughly investigated in the developing forebrain (for a review, see Alfano et al., 2013); however, only few data are available on COUP-TFI expression and function in the adult brain. Here, we address this issue focusing on the OB, a highly plastic region of the adult mammalian brain, characterised by continuous neurogenesis (Lledo et al., 2008). We show that in the adult OB, as in the cerebral cortex (Lodato et al., 2011), COUP-TFI is expressed in distinct subpopulations of GABAergic interneurons. Little or no staining was observed in migrating neuroblasts, indicating that COUP-TFI expression in the OB is mostly associated with mature cells integrated in the GcL and GL. Thus, although COUP-TFI seems to be required in GABAergic neuron tangential migration in the developing cortex (Tripodi et al., 2004), our expression data indicate that COUP-TFI is unlikely to be involved in adult SVZ neuroblast migration.

OB interneurons include multiple subtypes (Parrish-Aungst et al., 2007) that derive from spatially restricted progenitors (Bovetti et al., 2007; Ihie and Alvarez Buylua, 2011). We focused on the GL to identify the phenotype of COUP-TFI-expressing cells and found that they largely belong to the DAergic lineage. Notably, the large majority of PAX6+ and TH+ cells in the GL are found to be positive for COUP-TFI, supporting possible specific functions for COUP-TFI in olfactory DAergic neurons. Interestingly, we observed that late postnatal and adult DAergic cells express COUP-TFI in much higher percentages compared with early neonatal DAergic cells, strongly indicating COUP-TFI implication in features peculiar to the function of the adult DAergic circuit.

It is well established that a mixture of intrinsic determination mechanisms and activity-dependent cues are required for the DAergic phenotype acquisition and maintenance in the OB. Several transcriptional regulators, including PAX6, ER81, DLX2 and GSH2 (GSX2 – Mouse Genome Informatics) are involved in OB DAergic neuron generation and specification, whereas on the other side sensory input is necessary for maintaining TH expression in DAergic cells (Cave and Baker, 2009). Our data show that COUP-TFI is mainly involved in the maintenance of TH through an activity-dependent mechanism, as demonstrated in our genetic and functional experiments.

The Cre-LoxP conditional knockout mice in which COUP-TFI is selectively deleted in either the DLX5/6 or the EMX1 progenitor lineage show a specific downregulation of the TH+ population exclusively in the EMX1 lineage, without affecting other juxtaglomerular cell types (i.e. CR or CB+ cells). This was unexpected, as our previous analysis on cortical GABAergic interneuron populations, showed an imbalance between CR- and PV-expressing cortical interneurons in the absence of COUP-TFI in the DLX5/6 lineage (Lodato et al., 2011). Lack of any significant changes in COUP-TFIfl/flDlx5/6-Cre mice strongly suggest that this lineage only marginally contributes to COUP-TFI-expressing cells in the adult OB. Although we did not investigate whether COUP-TFI could act in other lineages involved in DAergic neuron generation (such as the GSH2 lineage) (Young et al., 2007), our data clearly show that absence of COUP-TFI in EMX1-expressing progenitors (Merkile et al., 2007; Ventura and Goldman, 2007) has a strong effect on adult TH-expressing cells. This could be because of a problem either in the generation/survival of DAergic cells or in the selective downregulation of TH expression in these cells. Our birthdating analysis showed no effect on the generation/survival of adult-born OB interneurons, but instead we found a reduction in the number of BrdU+ cells that were double positive for TH, in line with the observed decrease of TH+ cells over the whole GL interneuron population. Moreover, no differences were found in the density of PAX6+ and ER81+ cells, or in the percentages of PAX6+/BrdU+ and ER81+/BrdU+ cells, in the GL of COUP-TFIfl/flEmx1-Cre mice. Thus, COUP-TFI deletion affects TH expression independently from PAX6 and/or ER81 regulation, suggesting that interneurons committed to the DAergic phenotype still remain in the olfactory GL, but just fail to express TH.

Based on these genetic findings and on the observations of COUP-TFI sensory-dependent regulation in naris closure experiments, we hypothesised that this factor could be involved in mechanisms of activity-dependent modulation of TH expression. A direct effect of
COUP-TFI on TH expression regulation is unlikely, as no consensus sequences for COUP-TFI were found in the TH promoter sequence (our unpublished in silico data). Instead, we hypothesised that COUP-TFI could act through indirect mechanisms by controlling expression of genes related to odour-evoked responses, which in turn affect TH expression. Among the possible target genes, we focused our interest on the immediate early gene ZIF268, known as an activity-dependent regulator of inducible TH expression (Papanikolaou and Sabban, 2013).

Fig. 6. Lentiviral Cre-mediated deletion of COUP-TFI in adult-born juxtaglomerular cells decreases TH expression. (A-C) Experimental protocol. (A) Diagram of stereotaxic injections of lentiviral vectors expressing Cre recombinase and GFP (lenti-CRE-iGFP) or GFP only (lenti-iGFP) into the left and right hemispheres of adult COUP-TFIfl/fl mice, respectively. (B) Infected cells were analysed in OB GL 30 and 60 days post-injection (dpi). (C) Lentiviral injection triggers the infection of a subset of bulbar interneuron precursors in the rostral migratory stream (RMS, bottom panel). Infected cells are recognised as GFP+ (lenti-iGFP) or CRE+/GFP+ (lenti-CRE-iGFP) in ipsilateral OB GL (top panel) at the time of analysis. (D) Percentage of COUP-TFI+ or TH+ cells among the total number of infected cells 30 and 60 dpi in the OB GL ipsilateral to lenti-iGFP or lenti-CRE-iGFP injections. n=3-4 for each group. (E-H) Confocal images of the OB GL ipsilateral to lenti-iGFP injection stained for COUP-TFI (white, E), GFP (green, F) and TH (red, G). The arrow in H indicates a triple-labelled cell. (I-P) Confocal images of the OB GL ipsilateral to lenti-CRE-iGFP injection stained for COUP-TFI (white, I), GFP (green, J), and Cre recombinase (red, K). None of the injected cells (GFP+CRE+; arrows; J,K) expresses COUP-TFI (I), whereas triple-labelled GFP+/CRE+/TH+ cells can be observed (arrow; M-P). Insets in E-P show higher magnification with confocal re-slicing in H,L,P. Error bars indicate s.e.m. Student’s t-test; **P<0.01; ***P<0.001. Scale bar: 50 μm in P for E-P; 10 μm in insets in H,L,P.
inputs and supporting a link between COUP-TFI function and activity-dependent regulation of TH expression.

In conclusion, this study reveals a novel function for COUP-TFI in the mouse forebrain, and gives new insights into our understanding of the mechanisms regulating DAergic neurons in the adult OB. Among the multiple factors required for the specification and proper differentiation of OB DAergic neurons (Cigola et al., 1998; Cave and Baker, 2009; Akiba et al., 2010; Flames and Hobert, 2011; Banerjee et al., 2013), we propose that COUP-TFI acts selectively in adult-generated mature cells, where it cooperates to maintain TH expression in an activity-dependent manner (supplementary material Fig. S1).

**MATERIALS AND METHODS**

**Animals**

COUP-TFI-floxed (COUP-TFI<sup>fl/fl</sup>) mice were generated and genotyped as previously described (Armentano et al., 2007). These mice were either crossed to Dlx5/6-ires-Cre (Stenman et al., 2003) or to Emx1-ires-Cre (Gorski et al., 2002) to generate mice homozygous for COUP-TFI<sup>fl/fl</sup> and heterozygous for Dlx5/6-ires-Cre (COUP-TFI<sup>fl/fl</sup>,<sup>iCre</sup>) or Emx1-ires-Cre (COUP-TFI<sup>fl/fl</sup>,<sup>iCre</sup>). COUP-TFI<sup>fl/+</sup> or COUP-TFI<sup>fl/fl</sup> mice were used as controls, and the latter also for injections of lentiviral vectors. All lines were maintained in a C57BL/6J genetic background. TH-GFP mice carrying the GFP under the control of TH promoter (Sawamoto et al., 2001; Matsushita et al., 2002) were maintained as heterozygous by breeding with C57BL/6J inbred mice. C57BL/6J wild-type mice were used for olfactory deprivation (Charles River). C57BL/6J mice at postnatal (P) day 0, 7, 14 or 21 were also evaluated, together with a group of P7 COUP-TFI<sup>fl/fl</sup>,<sup>iCre</sup> mice. Animals were housed under a 12-hour light-dark cycle in an environmentally controlled room. 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 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.

**Olfactory deprivation**

Adult mice (8-week-old males; n=10) were lightly anaesthetised with a mixture of ketamine (Ketavet; Gellini) and xylazine (Rompun; Bayer) before inserting the nose plugs (polylethylene tubing, 0.7 mm) into the right naris for 42 days. Olfactory deprivation effectiveness was checked post-mortem by controlling that the nose plug was retrieved in the anterior part of the snout and by confirming decreased levels of TH expression in the OB through immunofluorescence (Baker et al., 1993). Animals in which the plug was not retrieved or failed to show decreased TH expression, were discarded.

**BrdU administration**

Adult mice (8-week-old males; n=14) received four injections, 4 hours apart, of BrdU (50 mg/kg in 0.1 M Tris, pH 7.4; Sigma) and were euthanised 42 days later.

**Viral production**

The lentiviral constructs include either an ires-GFP (lenti-iGFP) or a Cre recombinase-ires-GFP (lenti-CRE-iGFP) cassette under the control of a cytomegalovirus (CMV) promoter. Replication-incompetent lentiviral particles coated with vesicular stomatitis virus glycoprotein (VSVG) were packaged in 293T cells. Cells were transfected with 30 μg vector and packaging constructs, according to a conventional CaCl<sub>2</sub> transfection protocol. After 30 hours, medium was collected, filtered through 0.22 μm cellulose acetate and centrifuged at 20,000 rpm (50,000 g) for 2 hours at 20°C in order to concentrate the virus. The titer was evaluated by infecting 293T cells with serial dilution of the virus and assessing the lower concentration at which fluorescent cells were still detectable. In all preparations the virus titer was in the order of 10<sup>7</sup> ifu/ml.

**Stereotoxic injections**

Adult COUP-TFI<sup>fl/fl</sup> or C57BL/6J mice (8-12 weeks old, n=14) were used. Mice were anaesthetised with an intraperitoneal injection of a mixture of
Olfactory stimulation

Eight to 12-week-old COUP-TFI/+ or COUP-TFI/−;Emx1-Cre mice (n=13) were housed under standard conditions. The day before the experiment, animals were individually separated in acrylic filtering-covered cages to reduce background activity (Magavi et al., 2005). Before euthanasia, awake and alert mice (stimulated group, named ‘stim’) underwent the odor stimulation individually in a clear cage without food and water. A 30-minute exposure to a mixed set of natural odours [banana, lemon peel, basil, lavender, cloves, cinnamon; modified from previous studies (Magavi et al., 2005; Vincis et al., 2012)] was used to obtain a diffuse and scattered activation in the OB. Odours were placed in a tea ball hanging from the acrylic filtering cover. Animals were then placed in a clear standard breeding cage for an additional 30 minutes before euthanasia. Nonstimulated groups for each genotype (named ‘basal’) were treated under the same conditions except that the tea ball was left empty.

Tissue preparation

Postnatal (P7, P14, P21) and adult mice were anesthetised with an intraperitoneal injection of a mixture of ketamine (Ketavet; Gellini) and xylazine (Rompun; Bayer) and perfused transcardially with 0.9% saline solution, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. Brains were removed from the skull, postfixed for 6 hours in the same solution, cryoprotected in a 30% sucrose solution in 0.1 M PB, pH 7.4, frozen and cryostat sectioned (Leica Microsystems, Milan, Italy). Free-floating coronal serial sections (25 μm) were collected in multiwell dishes. Sections were stored at −20°C in antifreeze solution until use. P0 mice were anesthetised by hypothermia and their brains dissected and fixed by immersion overnight in a solution of 4% PFA in 0.1 M PB, pH 7.4. Specimens were then processed as described above except of cryostat coronal sections (14 μm), which were collected in series directly on glass slides.

Immunohistochemistry and immunofluorescence

Sections were incubated overnight at 4°C in primary antibody diluted in 0.01 M PBS (pH 7.4), 0.01-0.5% Triton X-100, and 1% normal serum of the same species as the secondary antibody, followed by 1 hour at room temperature (RT) in the appropriate secondary antibody. For BrdU immunostaining, sections were pre-treated with 2 N HCl and neutralised with borate buffer (pH 8.5) before anti-BrdU staining. Sections were then mounted on gelatine-coated slides, air dried, and coverslipped in polyvinyl alcohol with dibacilcyclo-octane (DABCO). For the avidin-biotin-peroxidase (ABC) method, sections were incubated for 1 hour at RT in secondary biotinylated antibody, followed by the avidin-biotin-peroxidase complex and developed by 3,3′-diaminobenzidine (DAB) and 0.0024% H2O2 in 0.05 M Tris-HCl, pH 7.6. Slices were mounted on gelatine-coated slides, air dried, and covered in DPX Mountant (Sigma-Aldrich). The following primary antibodies were used: anti-BrdU (1:5000, IFL; 1:10,000, BAS; rat; AbD Serotec), anti-caldibin D-28K (CB) (1:1000; rabbit; Swant), anti-CR (1:8000; rabbit; Swant), anti-CH (1:8000; mouse; Swant), anti-TH (1:2000; rabbit; Institut Jacques Boy, France), anti-TH (1:3000; mouse; Immunostar), anti-COUP-TFI (1:500; mouse; R&D Systems), anti-COUP-TFI (1:500; rabbit) (Tripodi et al., 2004), anti-PAX6 (1:3000; rabbit; Chemicon), anti-ER81 (1:15,000, IFL; 1:20,000, BAS; rabbit; a gift from T. Jessell, Columbia University, USA), anti-GFP (1:1000; chicken, Aves Lab), anti-DCC (1:500; goat, Santa Cruz), anti-NeuN (1:1000; mouse, Chemicon), anti-Cre recombinase (1:1500; rabbit; Covance), anti-ZIP268 (EGR1) (1:500; rabbit; Santa Cruz). Secondary antibodies were used as follows: anti-mouse, anti-rabbit and anti-rat Cy3-conjugated (1:300; Jackson ImmunoResearch); anti-rabbit and anti-mouse AlexaFluor 647-conjugated (1:600; Jackson ImmunoResearch); anti-goat, anti-rabbit and anti-rabbit biotinylated (1:250; Vector) followed by avidin-FITC incubation (1:400; Jackson ImmunoResearch) or avidin-biotin-peroxidase complex (Vector); anti-rabbit and anti-rat AlexaFluor 488-conjugated (1:400; Molecular Probes); anti-chicken AlexaFluor 488-conjugated (1:400; Jackson ImmunoResearch). Colocalization between COUP-TFI and GAD67 was analysed on adult GAD67-GFP mice expressing GFP under the control of the endogenous GAD67 gene promoter (Tammamaki et al., 2003), kindly provided by Prof. F. Rossi (University of Turin).

Microscopy and quantification

Cell counts were conducted blind. Cell counting and image analysis were performed on either a Nikon microscope coupled with a computer-assisted image analysis system (Neurolucida software, MicroBrightField), a Fluoview 500 confocal microscope (Olympus Instruments) or a TCS SP5 confocal microscope (Leica). Confocal image z-stacks were captured through the thickness of the slice at 1-μm optical steps and used for double- or triple-labelled cell counts. To estimate the volume of each layer, camera lucida drawings of sections were performed through the entire OB. The boundaries between layers were estimated from changes in cell density in sections stained with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI). The area of each section and layer was automatically calculated by Neurulucida software and the volume of the layers estimated applying the Cavalieri method (Prakash et al., 1994). Densities of positive cells were calculated applying a random sampling method using a virtual counting grid. 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. Cell density (D) was 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 layer area (μm²) and expressed as the number of positive cells per μm². Total cell number estimation (T) was calculated using the formula T=D×V/t where D is the cell density, V is the volume of the whole GL and t the thickness of the section analysed. Statistical comparisons were conducted by Student’s t-test, one-way or two-way ANOVA followed by Tukey post-hoc comparison, where appropriate. Significance was established at P<0.05. Cell counts and volumes are presented as mean ± s.e.m. and are derived from at least three different animals, analysing at least three OB sections (at anterior, medial and posterior levels) for each animal.

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Competing interests

The authors declare no competing financial interests.

Author contributions

S. Bovetti, S.D.M. and M.S. jointly coordinated and designed this work and wrote the manuscript. S. Bovetti, S.D.M. and S. Bonzano conducted experiments and analysis on COUP-TFI/+Emx1-Cre and COUP-TFI/−;Emx1-Cre mouse lines. S. Bovetti conducted BrdU and olfactory deprivation experiments and analysis; S. Bonzano and S.D.M. conducted viral injections and olfactory stimulation experiments; S. Bonzano performed confocal analysis and prepared the figures of the paper, D.G. and A.I. conducted immunohistochemical analysis; M.A. and M.S. contributed with generation of COUP-TFI/+ mice and tissue preparation from COUP-TFI/+ derived lines; S.G.G. produced the lentiviral vectors.

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Supplementary material

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