

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

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

Serena Bovetti^{1,*‡}, Sara Bonzano^{1,2,‡}, Donatella Garzotto¹, Serena Gea Giannelli³, Angelo Iannielli^{1,2}, Maria Armentano^{2,4}, Michèle Studer^{4,5,6} and Silvia De Marchis^{1,2,§}

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: Juxtglomerular 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 juxtglomerular

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 juxtglomerular 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 (Qiu 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

¹Department of Life Sciences and Systems Biology, University of Turin, Via Accademia Albertina 13, 10123, Torino, Italy. ²Neuroscience Institute Cavalieri Ottolenghi (NICO), Regione Gerzole 10, 10043, Orbassano, Italy. ³San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET), Division of Regenerative Medicine, Stem Cells, and Gene Therapy, San Raffaele Scientific Institute, Via Olgettina 28, 20132, Milano, Italy. ⁴Telethon Institute of Genetics and Medicine (TIGEM), Via P. Castellino 111, 80131, Napoli, Italy. ⁵University of Nice Sophia-Antipolis, Parc Valrose, 28 Avenue Valrose, F-06108 Nice, France. ⁶INSERM UMR 1091, Parc Valrose, 28 Avenue Valrose, F-06108 Nice, France.

*Present address: Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia (IIT), Via Morego 30, 16163, Genova, Italy.

[‡]These authors contributed equally to this work

[§]Author for correspondence (silvia.demarchis@unito.it)

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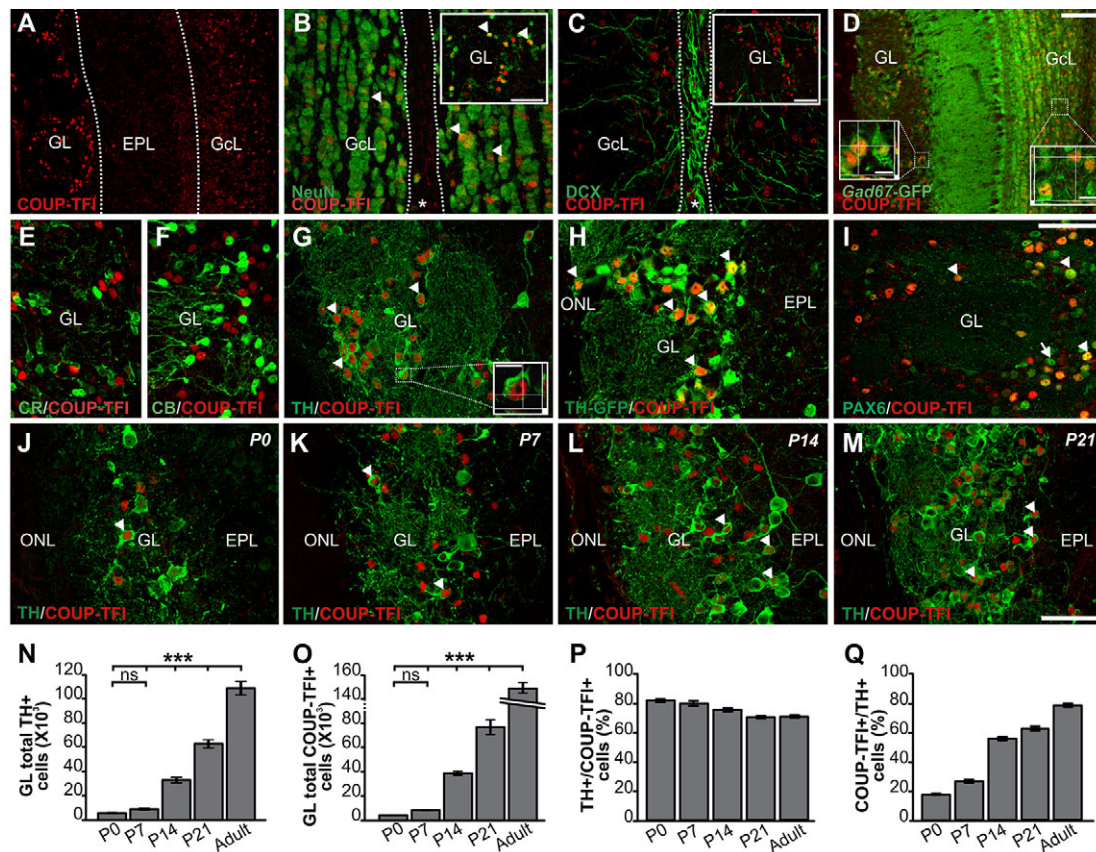


Fig. 1. COUP-TFI distribution in the adult and postnatal mouse OB. (A-D) Adult OB coronal sections stained in red for COUP-TFI and in green for NeuN (B), DCX (C) or GAD67-GFP (D) (GAD67 also known as GAD1 – Mouse Genome Informatics); insets in B and C show double-labelled cells in the GL; insets in D 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, $P\geq 0.05$. 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.

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 intense 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 for NeuN in the GcL ($85.7\pm 3.5\%$; $n=323$ cells) and nearly 52% of COUP-TFI⁺ cells did express NeuN in the GL ($52.4\pm 3.2\%$; $n=330$ cells). By contrast, COUP-TFI⁺ cells were rarely detected in the core of the OB (Fig. 1B,C, asterisks), where the migratory stream of DCX⁺ SVZ-derived neuroblasts enters the bulb (Fig. 1C). About 7% ($7.4\pm 1.7\%$; $n=586$ cells) and 4% ($3.7\pm 0.4\%$; $n=510$ cells) of COUP-TFI⁺ cells were double positive for DCX in the GcL and GL, respectively, indicating little COUP-TFI labelling in immature cells. Thus, COUP-TFI expression in the OB is mostly associated with mature neurons.

It is known that most cells in the GL and GcL are GABAergic interneurons (Parrish-Aungst et al., 2007). Analysis of *Gad67*-GFP mice (Tamamaki et al., 2003) (Fig. 1D) showed that virtually all COUP-TFI⁺ cells in the OB were co-labelled with green fluorescent

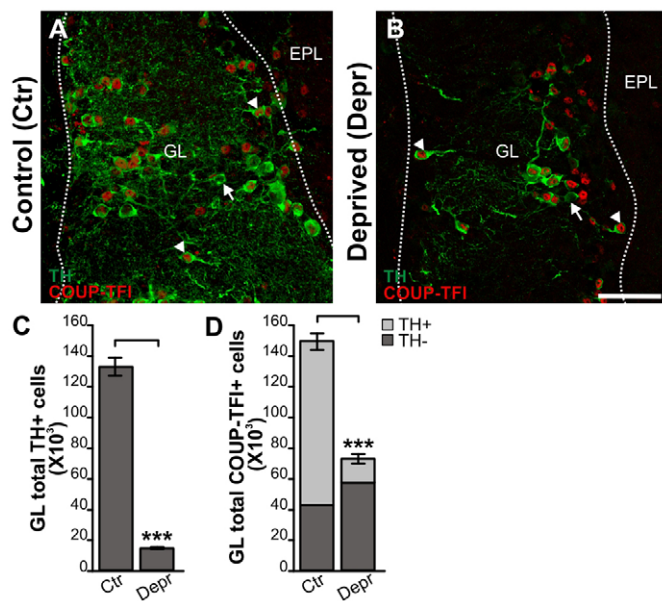


Fig. 2. Olfactory deprivation drastically decreases TH and COUP-TFI expression. (A) OB stained for COUP-TFI (red) and TH (green) in control (A) and deprived (B) mice 42 days following unilateral naris closure. (C) Number of TH⁺ cells in control and deprived mice. (D) Relative proportion of TH⁺ (light grey) and TH⁻ (dark grey) cells among the total number of COUP-TFI⁺ cells in control ($n=6$) and deprived ($n=4$) mice. Arrowheads: double labelled cells. Arrows: TH⁺/COUP-TFI⁻ cells. Error bars indicate s.e.m. Student's t -test; *** $P<0.001$ Scale bar: 50 μ m in B for A,B. EPL, external plexiform layer; GL, glomerular layer.

protein (GFP; $92.24 \pm 1.64\%$; $n=338$ cells); however, COUP-TFI⁺ cells represent only a subset of the GABAergic GFP⁺ cells. We thus focused on the GL to understand whether COUP-TFI expression was associated with specific neurochemical phenotypes by analysing double-labelled neurons for COUP-TFI and CR, CB or TH (Fig. 1E–G). Only a few cells were double labelled with COUP-TFI and CR or CB (CR⁺/COUP-TFI⁺: $0.96 \pm 0.21\%$; $n=591$ cells; CB⁺/COUP-TFI⁺: $2.15 \pm 0.20\%$; $n=284$ cells; Fig. 1E,F). However, ~71% of COUP-TFI⁺ cells were positive for TH (Fig. 1G,P, Adult). The fraction of COUP-TFI⁺ cells that were negative for TH might instead be juxtaglomerular cells of an unknown phenotype or, alternatively, might belong to DAergic precursor cells in which the transcription of the TH gene occurs with no significant translational activity (Baker et al., 2001). To test this latter hypothesis, we studied COUP-TFI distribution in the OB of a transgenic line expressing GFP under the TH promoter (Sawamoto et al., 2001; Matsushita et al., 2002) (Fig. 1H) and found that almost 83% of COUP-TFI cells were also GFP⁺ ($83.06 \pm 1.62\%$; $n=1537$ cells). In addition, all COUP-TFI⁺ cells in the adult OB GL were also positive for PAX6 ($n=516$ cells; Fig. 1I), a major determinant factor of the DAergic phenotype (Kohwi et al., 2005; Hack et al., 2005; de Chevigny et al., 2012). Notably, 90% of PAX6⁺ ($89.43 \pm 3.62\%$ $n=577$ cells) and nearly 80% of TH⁺ olfactory interneurons do express COUP-TFI (Fig. 1Q, Adult), indicating a strong association between this transcription factor and the DAergic phenotype.

Next, we analysed COUP-TFI expression in DAergic cells during OB postnatal development (postnatal ages: P0, P7, P14, P21; Fig. 1J–M). Quantification of TH⁺ cells in the developing OB showed a 20-fold increase from P0 to adult age (one-way ANOVA, $F_{(4,13)}=105.57$, $P<0.001$; Fig. 1N), with a drastic expansion of the

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 naris closure and the total number of TH⁺ and COUP-TFI⁺ cells was estimated in control (Ctr) and deprived (Depr) mice 42 days after naris 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 ($71.48 \pm 0.87\%$ in controls, $n=1558$ cells; $21.32 \pm 1.06\%$ 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., 2007; Young et al., 2007)] by crossing the *COUP-TFI*-floxed line (*COUP-TF^{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-TF^{fl/fl}/Dlx5/6-Cre* mice (one-way ANOVA, $F_{(2,10)}=82.845$, $P<0.001$. *fl/fl* versus *fl/fl^{Dlx5/6-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^{Dlx5/6-Cre}*, Tukey post-hoc $P=0.480$; Fig. 3B,E,K). On the contrary, 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,I), 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 ± 0.01 mm³ in *fl/fl* mice and 1.8 ± 0.06 mm³ in *fl/fl^{Emx1-Cre}* mice, t -test $P=0.46$).

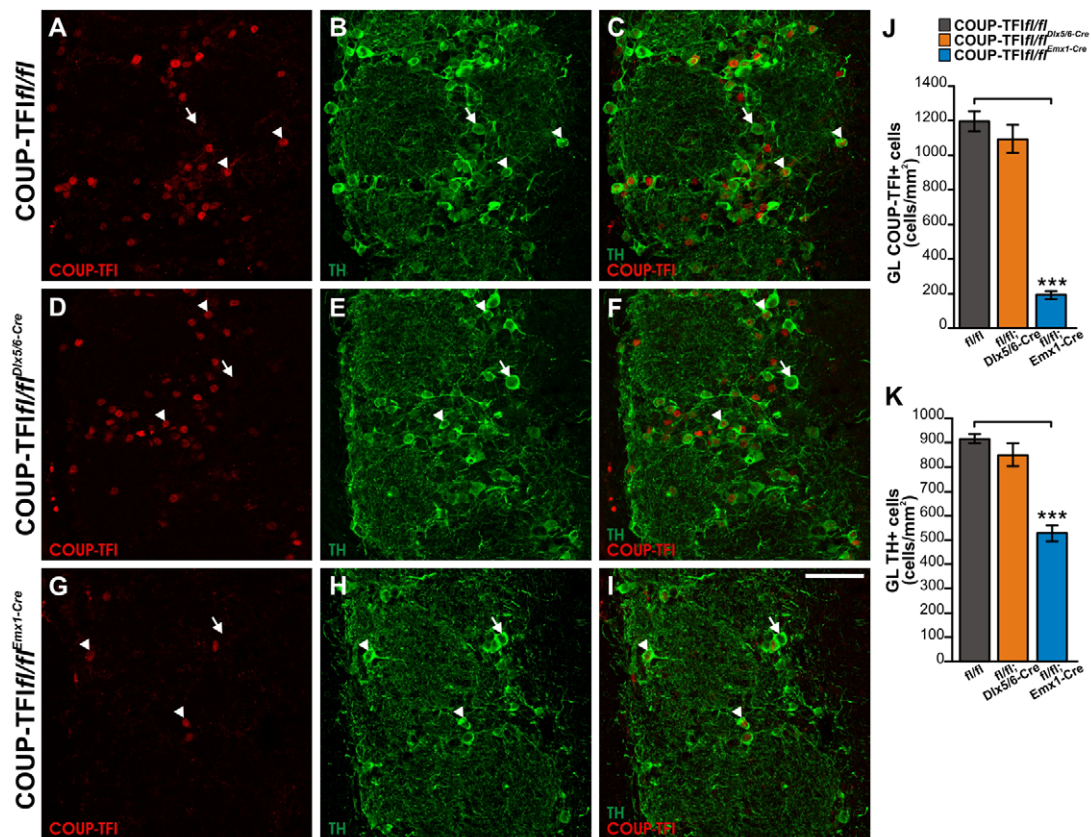


Fig. 3. Ablation of COUP-TFI in EMX1 lineage affects TH expression in the adult OB. (A-I) OB stained for COUP-TFI (red, A,D,G), and for TH (green, B,E,H), merged in C,F,I in *COUP-TFI^{fl/fl}* (control, A-C), *COUP-TFI^{fl/fl}Dlx5/6-Cre* (D-F) and *COUP-TFI^{fl/fl}Emx1-Cre* (G-I) adult mice. Scale bar: 50 μm in I for A-I. (J,K) Mean number of COUP-TFI+ cells (J) and TH+ cells (K) per squared millimetres in the GL of control (grey, *n*=5-6), *COUP-TFI^{fl/fl}Dlx5/6-Cre* (orange, *n*=4) and *COUP-TFI^{fl/fl}Emx1-Cre* (light blue, *n*=4) mice. Error bars indicate s.e.m. Tukey post-hoc; ****P*<0.001.

Importantly, analysis of TH immunofluorescence in the OB of *COUP-TFI^{fl/fl}Emx1-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-TFI^{fl/fl}Emx1-Cre* mice co-express COUP-TFI+ in a lower percentage compared with control mice ($26.56\pm1.73\%$ of TH+ cells were COUP-TFI+ in *fl/flEmx1-Cre*, *n*=496 cells; $80.81\pm0.36\%$ 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-TFI^{fl/fl}Emx1-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.

To further address the role of COUP-TFI in adult-generated DAergic cells, we injected bromodeoxyuridine (BrdU) in 8-week-old *COUP-TFI^{fl/fl}Emx1-Cre*, *COUP-TFI^{fl/fl}Dlx5/6-Cre* and control mice to label newborn interneurons (Fig. 4A-C). At 42 days survival post-BrdU injection, no changes in the overall BrdU+ cell density were observed in the GL among the different genotypes (one-way

ANOVA, $F_{(2,11)}=0.216$, $P=0.809$; Tukey post-hoc: *fl/fl* versus *fl/flDlx5/6-Cre* $P=0.825$; *fl/fl* versus *fl/flEmx1-Cre* $P=0.998$; *fl/flDlx5/6-Cre* versus *fl/flEmx1-Cre* $P=0.851$; Fig. 4A), indicating that lack of COUP-TFI does not affect adult-generated interneuron survival. Next, we quantified the percentage of BrdU+ cells that expressed TH in *COUP-TFI^{fl/fl}Emx1-Cre* mice and found a 36.4% reduction of double-labelled cells (*t*-test $P<0.05$; Fig. 4B,C), further supporting a role for COUP-TFI in the regulation of TH expression in adult-generated cells.

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+: $55.63\pm0.66\%$ in *fl/fl*, *n*=104 cells; $56.40\pm3.37\%$ in *fl/flEmx1-Cre*, *n*=99 cells; *t*-test $P=0.83$; ER81+/BrdU+: $54.77\pm0.28\%$ in *fl/fl*, *n*=115 cells; $59.37\pm3.69\%$ 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-TFI^{fl/fl}Emx1-Cre* mice showed no effect on these juxtglomerular populations (CB+: *t*-test $P=0.629$; CR+: *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.

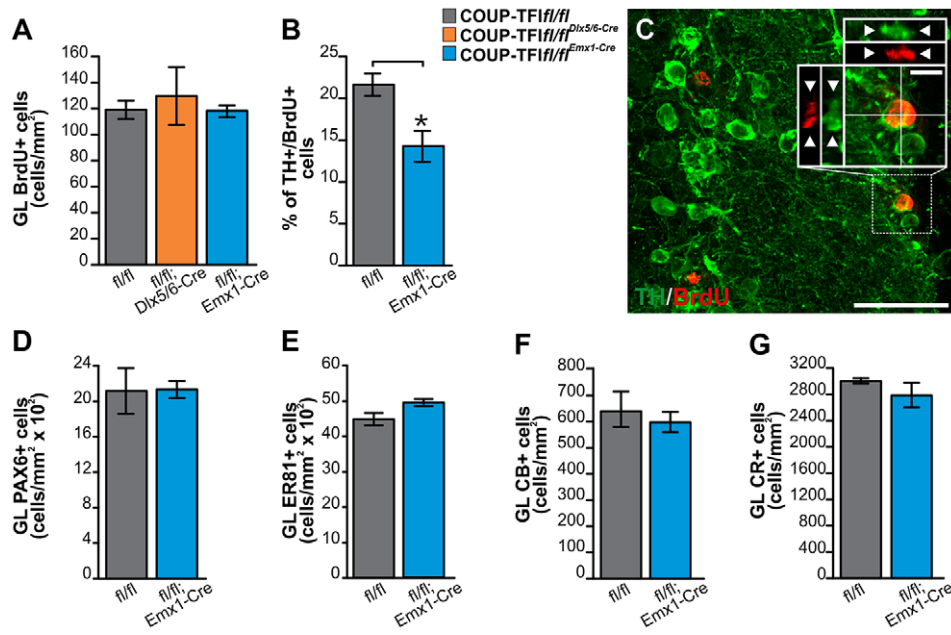


Fig. 4. Ablation of COUP-TFI does not affect adult-born interneuron survival and does not influence expression of OB interneuron markers except TH. (A) Mean number of BrdU⁺ cells per squared millimetre in the GL of control (grey, $n=7$), *COUP-TFI*^{fl/fl}*Dlx5/6-Cre* (orange, $n=4$) and *COUP-TFI*^{fl/fl}*Emx1-Cre* (light blue, $n=3$) mice 42 days following BrdU injection. (B) Percentage of BrdU⁺ cells double labelled for TH in the GL of control ($n=3$) and *COUP-TFI*^{fl/fl}*Emx1-Cre* ($n=3$) mice. (C) GL stained for TH (green) and BrdU (red). Inset shows a higher magnification of a re-sliced TH⁺/BrdU⁺ cell. (D–G) Mean number of Pax6⁺ (D), Er81⁺ (E) CB⁺ (F), CR⁺ (G) cells per squared millimetres in the GL of control ($n=3–4$) and *COUP-TFI*^{fl/fl}*Emx1-Cre* ($n=3–4$) mice. Error bars indicate s.e.m. Student's *t*-test; * $P<0.05$. Scale bar: 50 μ m in C; 10 μ m in inset.

Impaired ZIF268 expression in *COUP-TFI*^{fl/fl}*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-TFI*^{fl/fl}*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 through direct interactions with SP1 (Pipaón et al., 1999). Both *COUP-TFI*^{fl/fl}*Emx1-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-TFI*^{fl/fl}*Emx1-Cre* mice, we detected a lower density of juxtaglomerular cells expressing *ZIF268* compared with control mice in basal conditions (two-way ANOVA, $F_{(3,9)}=124.41$, $P<0.001$; basal *fl/fl* versus basal *fl/fl**Emx1-Cre*, Tukey post-hoc $P<0.001$; Fig. 5B–D). Although *COUP-TFI*^{fl/fl}*Emx1-Cre* mice responded to the odour mix stimulation with an increase in the density of *ZIF268*⁺ cells (basal *fl/fl**Emx1-Cre* versus stim *fl/fl**Emx1-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/fl**Emx1-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-TFI*^{fl/fl}*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-TFI^{fl/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-TFI*^{fl/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,I–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: $35.28\pm4.08\%$, $n=159$ infected cells; 60 dpi: $44.2\pm0.8\%$, $n=246$ infected cells), confirming the specificity of CRE activity in *COUP-TFI*^{fl/fl} mice. In lenti-iGFP injected *COUP-TFI*^{fl/fl} mice, the percentage of double-labelled TH⁺/GFP⁺ cells was about 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: $15.98\pm3.44\%$, $n=289$ infected cells; 60 dpi: $25.63\pm0.99\%$, $n=366$ 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: $43.4\pm2.1\%$, $n=208$ infected cells, lenti-CRE-iGFP: $45.6\pm3.5\%$, $n=177$ infected cells; *t*-test

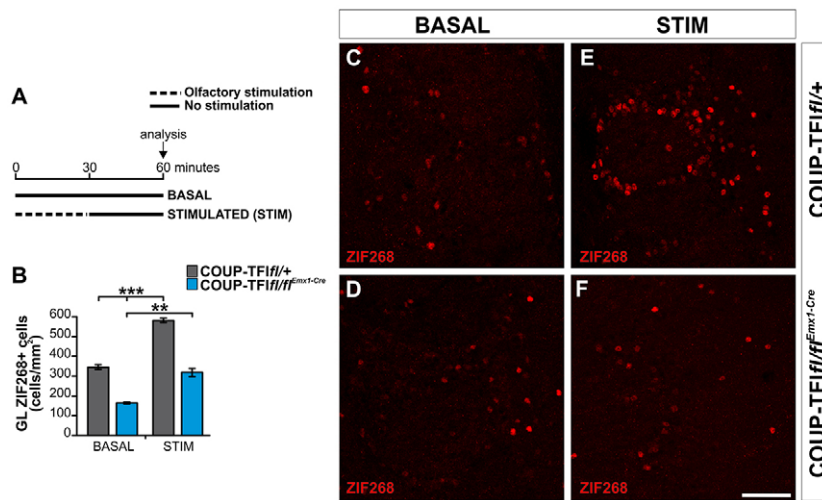


Fig. 5. Ablation of COUP-TFI in EMX1 lineage impairs ZIF268 induction. (A) Protocol of acute odour stimulation. (B) Mean number of ZIF268⁺ cells density in the GL of *COUP-TFII/+* (grey) or *COUP-TFII/ffEmx1-Cre* (light blue) mice in basal condition (BASAL) or following odour stimulation (STIM). (C-F) GL stained in red for ZIF268 in *COUP-TFII/+* (C,E) and *COUP-TFII/ffEmx1-Cre* (D,F) adult mice (BASAL in C,D or STIM in E,F). $n=3-4$ for each group. Error bars indicate s.e.m. Tukey post-hoc; ** $P<0.01$; *** $P<0.001$. Scale bar: 50 μ m in F for C-F.

$P=0.625$), indicating that CR⁺ cell type is not affected by the action of CRE.

Finally, we quantified the expression of ZIF268 in GFP⁺ and CRE⁺/GFP⁺ cells (Fig. 7) and found a statistically significant decrease of ZIF268⁺ cells in lenti-CRE-iGFP compared with lenti-iGFP injected mice (60 dpi, lenti-iGFP: $n=167$ infected cells; lenti-CRE-iGFP: $n=146$ infected cells; t -test $P<0.05$; Fig. 7D). Together, these results strongly support the argument for a cell-autonomous effect of COUP-TFI on the activity-dependent regulation of the DAergic phenotype.

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; Ihrie and Alvarez Buyla, 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 juxtglomerular 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-TFII/ff^{DLX5/6-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 (Merkle 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 birth-dating 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-TFII/ff^{Emx1-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

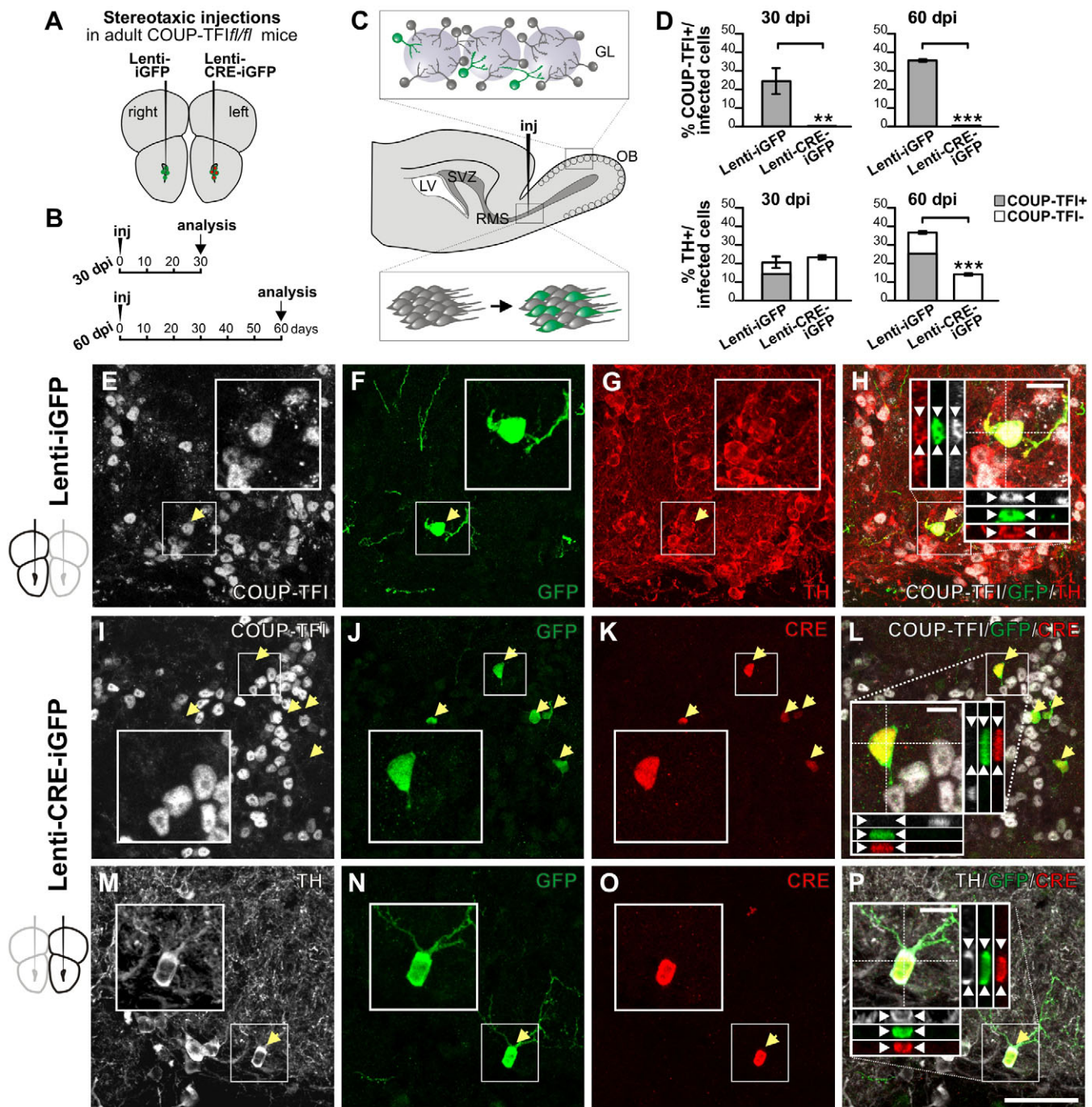


Fig. 6. Lentiviral Cre-mediated deletion of COUP-TFI in adult-born juxtglomerular cells decreases TH expression. (A-C) Experimental protocol. (A) Diagram of stereotaxic injections of lentiviral vectors expressing Cre recombinase and GFP (lenti-CRE-iGFP) or GFP only (lenti-iGFP) into the left and right hemispheres of adult COUP-TFI^{fl/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 in white for COUP-TFI (I) or TH (M), in green for GFP (J,N) and in red for Cre recombinase (K,O). 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 μ m in P for E-P; 10 μ m in insets in H,L,P.

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,

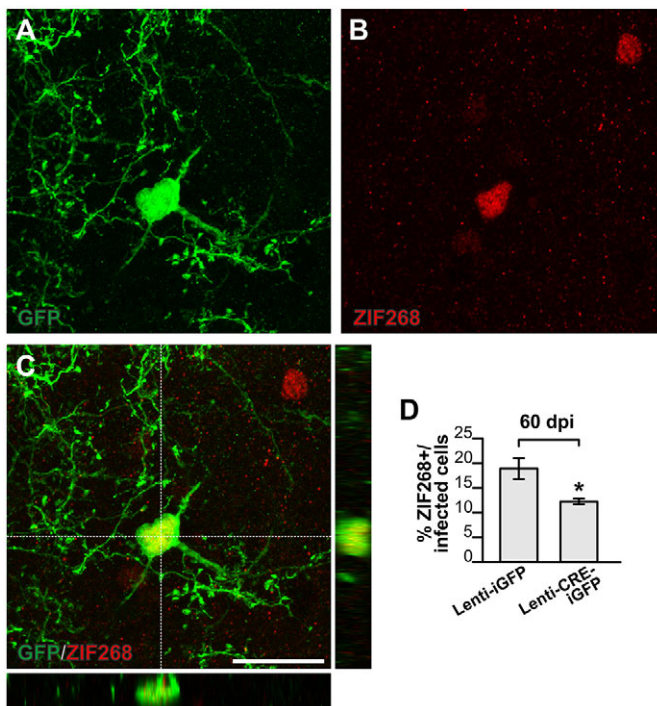


Fig. 7. Lentiviral Cre-mediated deletion of COUP-TFI decreases ZIF268 expression in adult-born juxtglomerular cells. (A–C) Representative confocal re-sliced image of a ZIF268⁺/GFP⁺ cell (C) stained for GFP (green, A) and ZIF268 (red, B) in the OB GL. (D) Percentage of ZIF268⁺ cell among the GFP⁺ infected cells in the OB GL at 60 dpi. *n*=3–4 for each group. Error bars indicate s.e.m. Student's *t*-test; **P*<0.05. Scale bar: 25 μm in C for A–C.

2000; Nakashima et al., 2003; Akiba et al., 2009; Kress and Wullmann, 2012; Bepari et al., 2012). We show that ZIF268 induction is severely impaired in the GL of COUP-TFI mutant mice, both in basal condition and following acute odour stimulation, supporting a role for COUP-TFI in the control of OB interneuron responsiveness. It is possible that COUP-TFI acts on ZIF268 promoter through interaction with other factors, such as SP1 (Rohr et al., 1997), which has been previously demonstrated to be involved in ZIF268 activation by COUP-TFI in other cellular systems (Pipaón et al., 1999). Further studies are needed to dissect the molecular interactions underlying ZIF268 expression regulation by COUP-TFI.

We further demonstrate that COUP-TFI regulation on TH expression is cell-autonomous by deleting COUP-TFI through lentiviral injections. After 30 days, absence of COUP-TFI in infected neurons has no effect on TH expression, confirming that COUP-TFI is not involved in the intrinsic determination of DAergic phenotype acquisition. However, longer survival time (i.e. 60 days) shows a net drop in the percentage of TH-expressing cells among the infected population. Although lenti-CRE-iGFP injection in *COUP-TFI*^{fl/fl} mice does not selectively target EMX1-derived progenitors, we initially showed that COUP-TFI-expressing juxtglomerular interneurons mainly derive from the EMX1 lineage and that TH downregulation upon COUP-TFI loss is restricted to this lineage. We thus infer that the drop in the percentage of TH-expressing cells among the lenti-CRE-iGFP infected population principally occurs in EMX1-derived juxtglomerular cells.

Overall, our results show that COUP-TFI is involved in the maintenance, rather than establishment, of the DAergic phenotype. In addition, GFP-infected cells at 60 dpi also show reduced ZIF268 expression, indicating impairment in cell responsiveness to sensory

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*^{fl/fl}) 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*^{fl/fl} and heterozygous for *Dlx5/6-IRES-Cre* (*COUP-TFI*^{fl/fl}^{Dlx5/6-Cre}) or *Emx1-IRES-Cre* (*COUP-TFI*^{fl/fl}^{Emx1-Cre}). *COUP-TFI*^{fl/+} or *COUP-TFI*^{fl/fl} 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*^{fl/fl}^{Emx1-Cre} 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 (polyethylene 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 CaCl₂ 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 assessed as the lower concentration at which fluorescent cells were still detectable. In all preparations the virus titer was in the order of 10⁷ ifu/ml.

Stereotaxic injections

Adult *COUP-TFI*^{fl/fl} or C57BL/6J mice (8–12 weeks old, *n*=14) were used. Mice were anaesthetised with an intraperitoneal injection of a mixture of

ketamine (Ketavet; Gellini) and xylazine (Rompun; Bayer) and placed on a stereotaxic apparatus. Bilateral injections into the RMS were performed at the following coordinates: mediolateral, ± 0.8 mm and anteroposterior, +3.1 mm to Bregma; and -2.9 mm from the pial surface (modified from Pallotto et al., 2012). Each mouse received two injections: a control injection in the right RMS (lenti-iGFP) and a Cre-mediated COUP-TFI ablating injection in the left RMS (lenti-CRE-iGFP). Viral vector particles (diluted 1:4 into 1 μ l 0.9% saline solution) were slowly injected using a glass micropipette and a pneumatic pressure injection apparatus (Picospritzer II, General Valve Corp., Fairfield, IL, USA). Animals were then placed into breeding standard cages and monitored until they resumed feeding and grooming activity, and euthanised 30 or 60 dpi.

Olfactory stimulation

Eight- to 12-week-old *COUP-TFII^{fl/+}* or *COUP-TFII^{fl/fl}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 odour 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 anaesthetised 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 μ m) were collected in multiwell dishes. Sections were stored at -20°C in antifreeze solution until use. P0 mice were anaesthetised 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 μ m), 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 of the secondary antibody, and then incubated for 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 diazabicyclo-octane (DABCO). For the avidin-biotin-peroxidase (BAS) method, sections were incubated for 1 hour at RT in secondary biotinylated antibody, followed by the avidin-biotin-peroxidase complex and developed by incubation in 0.015% 3,3'-diaminobenzidine (DAB) and 0.0024% H_2O_2 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-calbindin D-28K (CB) (1:1000; rabbit; Swant), anti-CR (1:8000; rabbit; Swant), anti-CR (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-DCX (1:500; goat, Santa Cruz), anti-NeuN (1:1000; mouse, Chemicon), anti-Cre recombinase (1:1500; rabbit; Covance), anti-ZIF268 (EGR1) (1:500; rabbit;

Santa Cruz). Secondary antibodies were used as follows: anti-mouse, anti-rabbit and anti-rat Cy3-conjugated (1:800; Jackson ImmunoResearch); anti-rabbit and anti-mouse AlexaFluor 647-conjugated (1:600; Jackson ImmunoResearch); anti-goat, anti-rat 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 (Tamamaki 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- μ m 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 NeuroLucida 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 (μm^2) and expressed as the number of positive cells per mm^2 . 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 \pm 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-TFII^{fl/fl}Emx1-Cre* and *COUP-TFII^{fl/fl}Dlx5-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-TFII^{fl/fl}* mice and tissue preparation from *COUP-TFII^{fl/fl}* derived lines; S.G.G. produced the lentiviral vectors.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.089961/-/DC1>

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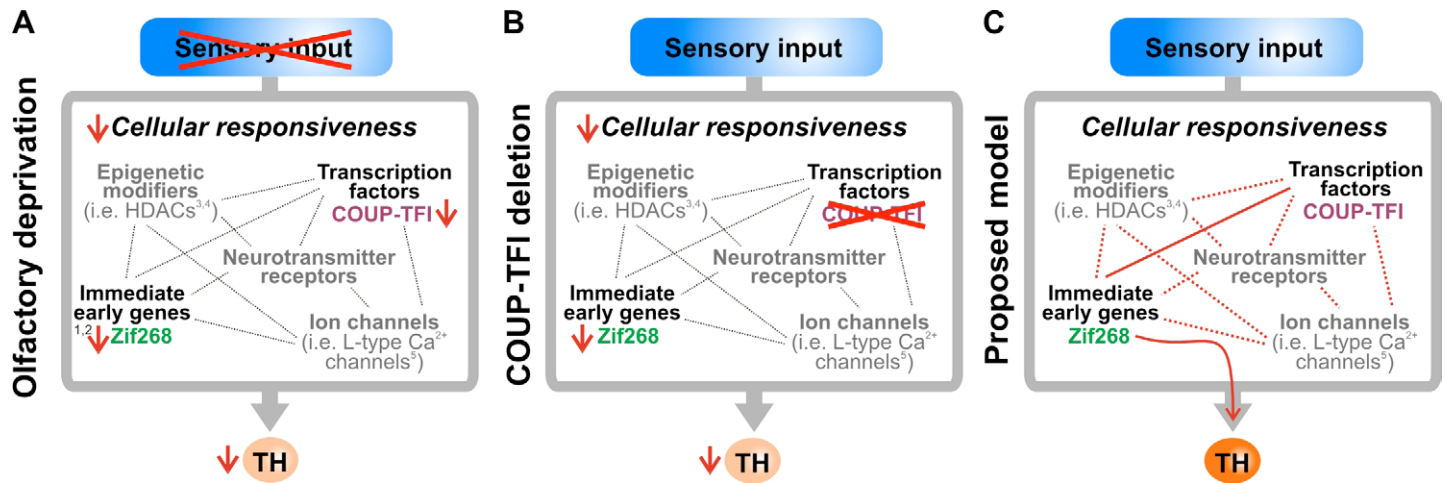


Fig. S1. Model of COUP-TFI involvement in activity-dependent TH expression in olfactory DAergic neurons. (A) Olfactory deprivation triggers a downregulation of COUP-TFI, Zif268¹ and TH expression in OB GL. (B) Loss of COUP-TFI function decreases both Zif268 and TH in presence of normal sensory input. (C) Proposed model for COUP-TFI role in sensory-dependent regulation of TH expression in the adult OB: COUP-TFI controls the cellular responsiveness to sensory input by regulating either directly and/or indirectly the activity of immediate early genes such as Zif268 and in turn influences TH expression. ¹Akiba et al., 2009; ²Kress and Wullimann, 2012; ³Akiba et al., 2010; ⁴Banerjee et al., 2013; ⁵Cigola et al., 1998.