

NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis

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After their generation and specification in periventricular regions, neuronal precursors maintain an immature and migratory state until their arrival in the respective target structures. Only here are terminal differentiation and synaptic integration induced. Although the molecular control of neuronal specification has started to be elucidated, little is known about the factors that control the latest maturation steps. We aimed at identifying factors that induce terminal differentiation during postnatal and adult neurogenesis, thereby focusing on the generation of periglomerular interneurons in the olfactory bulb. We isolated neuronal precursors and mature neurons from the periglomerular neuron lineage and analyzed their gene expression by microarray. We found that expression of the bHLH transcription factor NeuroD1 strikingly coincides with terminal differentiation. Using brain electroporation, we show that overexpression of NeuroD1 in the periventricular region *in vivo* leads to the rapid appearance of cells with morphological and molecular characteristics of mature neurons in the subventricular zone and rostral migratory stream. Conversely, shRNA-induced knockdown of NeuroD1 inhibits terminal neuronal differentiation. Thus, expression of a single transcription factor is sufficient to induce neuronal differentiation of neural progenitors in regions that normally do not show addition of new neurons. These results suggest a considerable potential of NeuroD1 for use in cell-therapeutic approaches in the nervous system.

adult neurogenesis | bHLH transcription factor | interneurons | postnatal electroporation

Determination of neuronal subtypes is an early event that coincides with cell cycle exit (1, 2). However, after their generation, new neurons have to remain immature for prolonged periods, allowing their migration to final destinations where terminal differentiation occurs (3). Little is known about the factors that maintain the precursor state or induce terminal differentiation.

Olfactory neurogenesis is particularly suited to approach these late steps in neuronal differentiation. Here, stem cell populations first located in the ventricular zone and after the establishment of an ependymal layer positioned in subventricular zone (SVZ) generate migratory neuroblasts throughout life (4). These perform long-distance chain migration via the rostral migratory stream (RMS) into the olfactory bulb (OB), where they migrate into the granule cell layer (GCL) and the glomerular layer (GL) to differentiate into GABA- and dopaminergic neurons (4, 5). Thus, in this system, generation of neurons is permanent and the consecutive steps in the neurogenic sequence are spatially separated.

Determination of newly generated neurons has been studied intensively over the past years. For example, it has been demonstrated that defined areas surrounding the lateral ventricle contain predetermined stem cells that give rise to defined subsets of interneurons (6, 7). Several transcription factors have been implicated in the specification of the different neuronal populations. The zinc finger transcription factor *sp8*, for instance, appears to be involved in the generation of interneurons expressing calretinin (8), and analysis of *Sall3* mutant mice (9)

points to a role of this factor in the dopaminergic, tyrosine hydroxylase-positive lineage (10). Furthermore, it appears that interneuron diversity relies on the combinatorial expression of such transcription factors. This is exemplified by *Pax6* and *Dlx2*, which have been shown to interact in the determination of adult generated interneurons toward a dopaminergic fate (9, 11, 12). All of these transcriptional regulators are expressed early during the neurogenic process and remain present until terminal differentiation occurs.

We aimed at the identification of transcription factors that induce terminal differentiation of postnatal generated neurons in the OB. To do so we isolated neuronal precursors and differentiated interneurons from the periglomerular lineage of the OB and compared their gene expression by microarray. We established that the expression of NeuroD1, a bHLH transcription factor that has been implicated in neuronal differentiation in several experimental systems (13–17), coincides with the passage from neuronal precursor to mature interneurons. Functionally, we show that premature expression of NeuroD1 *in vitro* and *in vivo* induced highly efficiently the differentiation of forebrain progenitors. *In vivo*, this leads to the transitory appearance of ectopic neurons in the SVZ, RMS and striatum. Conversely, knockdown of NeuroD1 specifically inhibits terminal maturation of periglomerular neurons in the OB. Thus, NeuroD1 is both necessary and sufficient to induce key steps in terminal neuronal differentiation.

Results

NeuroD1 Is Specifically Expressed in Mature GL Interneurons. Subpopulations of neuronal precursors destined for the GCL and GL of the OB are generated by regionally defined stem cell populations in the periventricular region but migrate intermingled in the RMS to the OB. Once there, cells reaggregate: granule cell precursors terminate their migration in the GCL, whereas the smaller population of periglomerular neuron precursors traverses this layer and the mitral cell layer (MCL) to invade the peripherally located GL (Fig. 1A). Thus, at a given time point, the GL contains both mature periglomerular neurons and their specific progenitors. Based on this spatial organization we isolated these two populations, concurrently depleting glial cells.

We devised a three-step strategy based on the following: (i) microdissection followed by enzymatic dissociation of the post-

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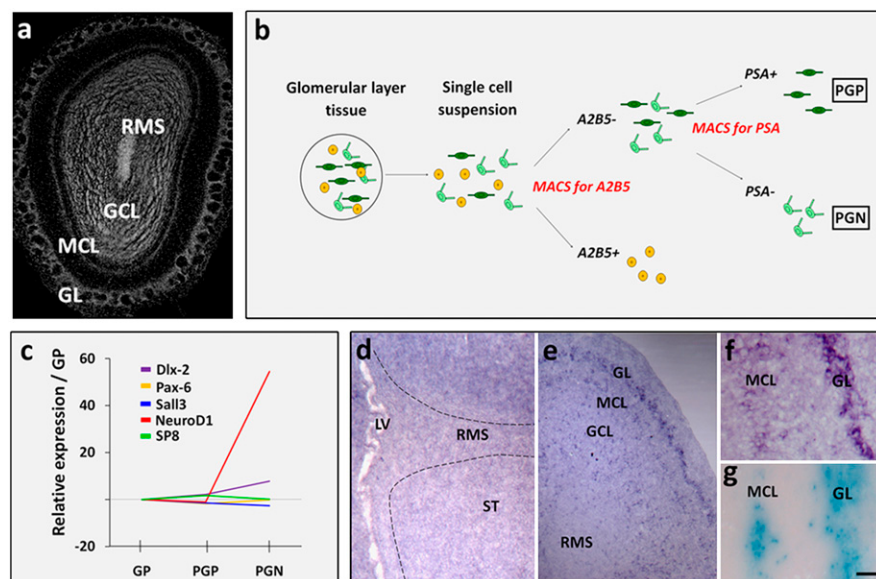


Fig. 1. Expression of NeuroD1 in the olfactory neurogenic system. (A) DAPI-stained coronal section through the olfactory bulb of P5. (B) Strategy to isolate neuronal populations at different steps of their maturation. (C) Relative changes in gene expression for selected genes. Expression in GP was considered baseline, and changes are expressed as fold difference. (D–F) NeuroD1 in situ hybridization on sections from P5 mouse brain. No signal was detected along the lateral ventricle or in the RMS (D). In the olfactory bulb, individual NeuroD1+ cells were present in the GCL, whereas the MCL and the GL contained higher amounts (E, high magnification in F). A similar expression pattern was found after β -gal reaction on NeuroD1-lacZ-knockin tissue (G). (Scale bar: 200 μ m in A; 100 μ m in D and E; 20 μ m in F and G).

natal GL, (ii) depletion of contaminating glial cells by magnetic activated cell sorting (MACS) using an A2B5 specific antibody (18), (iii) separation of PSA-NCAM expressing cells (19) from the remaining fraction containing the mature neurons (Fig. 1B). The same purification strategy was applied to tissue microdissected from the P2 periventricular region (18). Characterization of the different cell population after sorting was performed via immunocytochemistry using the markers used for sorting (A2B5 and PSA-NCAM) as well as the differentiation marker Gad65 (18) (Fig. S1). Thus, as starting material we obtained highly enriched mature OB periglomerular interneurons (PGN), their immature progenitors (PGP), as well as a mixed population of generic progenitors (GP) from the SVZ/RMS.

Based on the purified and characterized cell populations, we performed microarray analyses to gain insight into the changes in gene expression during the neurogenic process. Investigation of expression dynamics of genes associated with either the precursor status or neuronal differentiation (Fig. S2A and B) were used to validate the approach. Furthermore, these data were compared with those from an already available Serial Analysis of Gene Expression (SAGE) study (20).

Serial Analysis of Microarray (SAM) demonstrated the presence of groups of genes with comparable expression patterns (Fig. S2C–E). Interestingly, only a relatively small fraction of genes were absent in the immature cell populations GP and PGP but highly represented in mature PGN (Fig. S2E). One of the genes showing such a pattern was NeuroD1, which was expressed more than 50-fold higher in PGN than in the immature populations (Fig. 1C). This was in agreement with the above-cited SAGE data, showing that NeuroD1 expression was below the detection level in neuronal precursors of the adult SVZ (20). Thus, expression of NeuroD1 was absent from precursors but coincided with terminal neuronal differentiation.

This late expression of NeuroD1 was in contrast to that of factors that have been functionally implicated in the specification of PGN, including Pax6, Sp8 and Sall3, which were expressed in both the immature populations and in the mature neurons (Fig. 1C; in situ hybridization for Pax6 in Fig. S3). Only Dlx2 showed a moderate increase in the PGN lineage outgoing, however, from an already considerable baseline level in migrating precursors (12) (Fig. 1C).

Next we analyzed the expression of NeuroD1 using in situ hybridization on P5 forebrain sections. Strong expression was found in the GL, whereas weaker expression was observed in the GCL and MCL (Fig. 1E and F). The transcript was undetectable

in the periventricular region and the RMS (Fig. 1D). This staining was confirmed using NeuroD1-lacZ knockin mice (21) (Fig. 1G). In conclusion, these data demonstrated the absence of NeuroD1 from immature cells of the system and its strong expression in mature PGN. This pattern was coherent with a function in terminal neuronal differentiation.

NeuroD1 Induces Neuronal Differentiation in Vitro. We studied the neurogenic potential of NeuroD1 in primary cultured neural stem cells using the neurosphere assay. In parallel to NeuroD1, we performed all experiments under the same conditions using the transcription factor Pax6, a well-described neurogenic signal in the system (9, 11, 12), to control for specificity of the observed effects. Neurosphere cells were coelectroporated with NeuroD1 or Pax6 expression vectors and GFP immediately before plating in differentiation conditions. One week after transfection, in the control condition, $14 \pm 1\%$ of the GFP-positive cells coexpressed the early neuronal marker Tuj1 (Fig. S4A and D) whereas NeuroD1 induced Tuj1 expression in virtually all cells ($98.0 \pm 2\%$, Fig. S4B and D). Pax6 gain-of-function led to an intermediate value ($60.0 \pm 3\%$, Fig. S4C and D). NeuN, a later neuronal marker (22), was expressed by $21.1 \pm 1\%$ of the Tuj1-positive cells in the control situation (Fig. S4E and H) but induced by NeuroD1 in almost all cells ($93.9 \pm 2\%$; Fig. S4F and H). Surprisingly, Pax6 expression led to nearly complete disappearance of NeuN ($1.7 \pm 0.3\%$; Fig. S4G and H). We investigated the induction of subtype specific markers by NeuroD1. Whereas tyrosine hydroxylase showed no augmentation, we found a 20% increase in calretinin labeling, in agreement with previous findings (23).

Next we investigated morphological parameters like process length as well as density and length of filopodia. Both NeuroD1 and Pax6 induced a significant, greater than 2-fold increase in process length (Fig. S4I and L). We analyzed dendritic filopodia, structures that are believed to be precursors of dendritic spines (24). Expression of NeuroD1 induced a doubling in density and length of filopodia (Fig. S4N, P, and Q). Interestingly, Pax6 reduced filopodia density to a level significantly below that of controls (Fig. S4O and P), whereas length of the few remaining filopodia was not affected ($7.0 \pm 0.4 \mu$ m; Fig. S4Q).

Thus, the expression of NeuroD1 in neurosphere amplified neural stem cells induced neuronal commitment as well as morphological characteristics of mature neurons. Like NeuroD1, Pax6 favored neuronal commitment but appeared to actively suppress certain characteristics of terminal neuronal differentiation.

NeuroD1 Induces Ectopic Neurons in Vivo. We asked whether NeuroD1 was also sufficient to induce neuronal differentiation in vivo. We used postnatal forebrain electroporation, an approach that allows efficient genetic manipulation of neural stem cells along the lateral ventricles and, consequently, of all transitory or permanent cell populations that are generated in the olfactory neurogenic process (25). The NeuroD1 expression vector or empty control plasmids were coelectroporated together with a GFP-containing vector that allowed visualization of transfected cells and their progeny at high resolution. Consequences of NeuroD1 gain-of-function were analyzed at 2, 4, 6, 8, and 15 days post-electroporation (dpe). As for the in vitro studies, results were compared with the effects of Pax6 gain-of-function.

At 2 dpe of a control vector into the lateral wall of the forebrain ventricle, $9.8 \pm 1.3\%$ (Fig. 2*A* and *K*) of the GFP-expressing cells were localized in the VZ and had the morphology of radial glia (RG) (25). The majority of the GFP + cells, representing mainly neuronal precursors, were localized in the SVZ. Electroporation of a NeuroD1 expression vector induced a loss of GFP-positive RG cells ($3.7 \pm 0.5\%$; Fig. 2*B* and *K*). The remaining cells in the

VZ showed lower GFP levels than in controls (Fig. 2 *A* and *B*, asterisks).

At 4 dpe, in the control situation, considerable amounts of strongly GFP+ RG cells were still present in the VZ (Fig. 2C, asterisks), whereas NeuroD1 expression induced an almost complete loss of RG cells (Fig. 2D and K). At this time point, control cells were found along the entire SVZ and RMS. They showed generally tangential orientation and the typical morphology of migratory neuronal precursors. Large amounts of such cells were also found in the center of the OB (Fig. 2G and L). NeuroD1 expression induced an accumulation of GFP-labeled cells in the SVZ (Fig. 2D and L) at the expense of cells in the RMS (Fig. 2H, quantified in Fig. 2L). The accumulating cells did not have the appearance of migrating precursors but displayed complex multibranched morphologies (Fig. 2F and F', examples in Fig. 2I and I', quantified in Fig. 2M). All principal processes of these cells were covered with small protrusions resembling filopodia (Fig. 2J). Such morphologically complex cells, strongly resembling neurons, were also predominant in and along proximal parts of the RMS (Fig. 2F'). Interestingly, considerable amounts

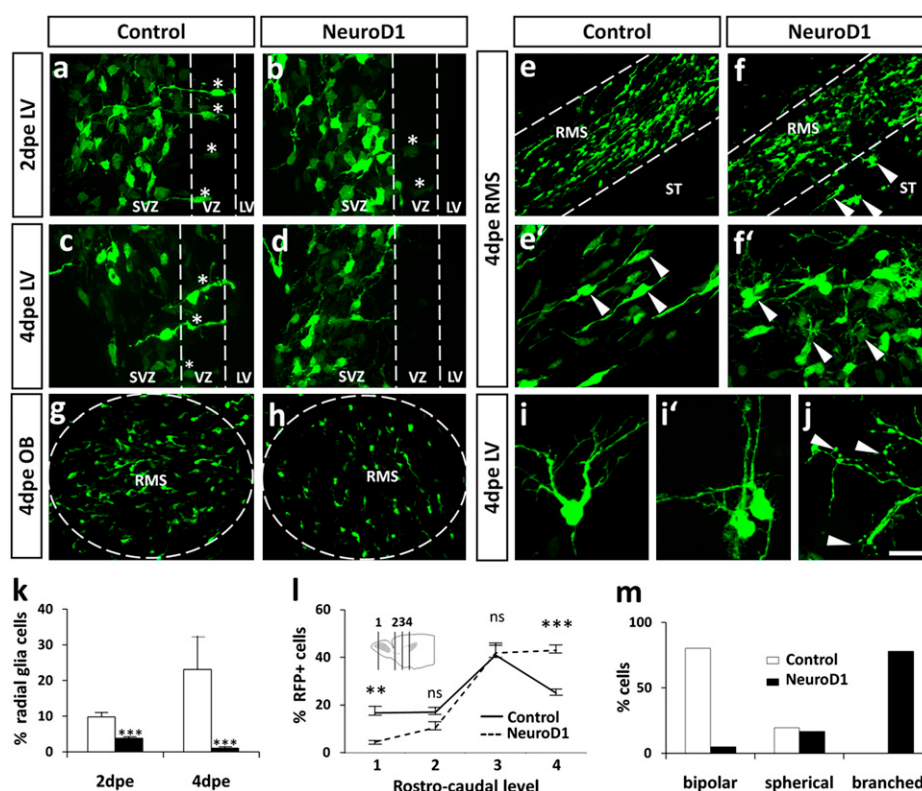


Fig. 2. NeuroD1 induces neuronal morphology in vivo. Effect of NeuroD1 gain-of-function at different time points postelectroporation. (A and B) Coronal forebrain sections at the level of the lateral ventricle at 2 dpe. In the control condition, strongly GFP labeled RG are present in the VZ (A, asterisk). Expression of NeuroD1 induced a relative loss of radial glia and fainter GFP label (B, asterisk). (C and D) Coronal sections at the level of the lateral ventricle at 4 dpe. NeuroD1 expression induced an accumulation of transfected cells in the SVZ (D) and the almost total disappearance of radial glia (D). (E–F) Sagittal sections of the RMS at 4 dpe. In the control situation, cells migrated toward the OB and presented the bipolar morphology specific of migrating precursors (E, E', arrowheads). NeuroD1 electroporation induced loss of tangential orientation, induction of complex branching (F, F', arrowhead), and invasion of the surrounding tissues (F, arrowheads). (G and H) Coronal section at the level of the olfactory bulb at 4 dpe. Although the majority of cells have reached the OB in the control situation (G), only a few cells were located in the center of the OB in the presence of NeuroD1 (H). (I and I') Examples of cells presenting neuronal morphology in the SVZ at 4 dpe. (J) High magnification showing the presence of filopodia covering NeuroD1-expressing cells (arrowheads). (K) Quantification of GFP-positive cells presenting radial glia cell morphology along the lateral ventricle at 2 and 4 dpe. Control: $9.8 \pm 1.3\%$ ($n = 6$) at 2 dpe; $24 \pm 11.8\%$ at 4 dpe ($n = 3$); NeuroD1: $3.7 \pm 0.5\%$ at 2 dpe ($n = 6$); $1.6 \pm 0.7\%$ at 4 dpe ($n = 3$). (L) Distribution of the GFP-positive cells along the rostrocaudal axis. NeuroD1-expressing cells accumulated in proximal parts of the system. (M) Morphological analysis of cells in the SVZ/RMS. Three different classes were defined: (i) bipolar cells presenting tangential orientation, (ii) spherical cells, and (iii) branched cells presenting multiple processes in various directions (compare I). NeuroD1-expressing cells presented a highly branched morphology. Control: bipolar, 80.4%; spherical, 19.5%; branched, 0% ($n = 133$ cells). NeuroD1: bipolar, 5%; spherical, 16.8%; branched, 78% ($n = 119$ cells). Statistics: Mann-Whitney test. ns, not significant. $**P < 0.01$; $***P < 0.005$. (Scale bar: 100 μm in E, F, G, and H; 25 μm in A, B, C, D, E, and F; 10 μm in I; 5 μm in J.)

of multibranched cells were found outside of the periventricular region and the RMS, invading neighboring structures such as the striatum (Fig. 2*F*, arrows). There was a clear correlation between the quantity of transgene expression, as visualized by GFP fluorescence, and the above parameters. Thus, NeuroD1 induced dose-dependently a neuron-like morphology in cells in the SVZ, RMS, and surrounding tissues.

We characterized the NeuroD1 induced neuron-like cell population in the periventricular region using neuronal and glial markers (Fig. 3; examples in Fig. S5). Doublecortin (DCX), a microtubule-associated protein expressed in migratory neuronal precursors (26), was seen in $75.2 \pm 4.5\%$ of the cells in the control situation but showed a significant increase after expression of NeuroD1 ($91.7 \pm 2.2\%$). NeuN, a marker for most mature neuronal cell types in the brain (22) was low in controls ($5.2 \pm 1.4\%$, $n = 8$) but strongly induced by NeuroD1 ($65.9 \pm 4.5\%$, $n = 9$). Map2, a later generic neuronal marker (27), was also rare in control cells ($14.1 \pm 1.4\%$, $n = 3$) but highly expressed in the NeuroD1 condition ($61.9 \pm 2.7\%$, $n = 3$). GFAP and Olig2 did not show significant alterations due to NeuroD1 expression. Thus, the NeuroD1-induced ectopic cells with neuronal morphology in the SVZ and RMS showed molecular characteristics of neurons.

Interestingly, whereas in the control situation at 8 dpe large amounts of strongly GFP positive neurons could be observed in the GCL and GL of the OB, in the NeuroD1 condition, GFP-positive cells in the OB were rare and less complex (25) (Fig. S6*A* and *B*). At this time point, very few surviving cells, generally expressing NeuN, were observed in the periventricular region (Fig. S6*C* and *C'*). At 15 dpe, the NeuroD1 electroporated brains were devoid of GFP (Fig. S6*D* and *E*).

We compared these findings to the consequences of Pax6 expression in the postnatal periventricular region. At 2 dpe, repartition and GFP expression in Pax6-expressing cells in the VZ was indistinguishable from that in controls (Fig. S7*A* and *B*; compare Fig. 2*A* and *K*). At 4 dpe, morphology of cells migrating in the SVZ and RMS was precursor-like and similar to that in controls. Complex neuron-like cells were not observed (Fig. S7*D* and *E*). Distribution of cells over the entire SVZ–RMS–OB axis was not affected (Fig. S7*F*). Although we did not test the entire panel of markers in Pax6 overexpression experi-

ments, we found that neither DCX (Fig. S7*G*, *H*, and *K*) nor NeuN (Fig. S7*I* and *K*) showed any alterations in this situation. At 15 dpe, Pax6-expressing neurons were found in the OB indistinguishable from those in the controls (25). An increase in TH-expressing neurons, as described before (11, 12), was not observed at this early time point. Later time points were not analyzed because of the transitory expression of the transgene. In conclusion, comparison of the effects of NeuroD1 to Pax6 demonstrated that the neuron-inducing activity in the periventricular region was a specific property of NeuroD1.

Next, we investigated whether NeuroD1 expression was associated to the induction of markers for nonoverlapping specific subpopulations of PGNs (28). Because the appearance of specific neuronal markers occurs after the induction of generic markers (5), these analyses were performed at 6 dpe. The calcium-binding proteins calbindin and parvalbumin, as well as the key enzyme for dopamine synthesis, tyrosine hydroxylase (TH), did not show an increase caused by gain-of-function of NeuroD1 (Fig. S8*B*). A small but significant population of cells was found to express calretinin after electroporation of NeuroD1 (Fig. S8*A* and *B*). However, the expression at the cellular level was weak compared with the natural expression of calretinin, for example, in the GL.

NeuroD1 Is Necessary For OB Interneuron Differentiation in Vivo.

Next we asked whether NeuroD1 is essential for the generation of PGN. Given that NeuroD1 deficiency in mice is generally associated with perinatal lethality (14, 15, 21), we used a strategy based on RNAi in concert with postnatal in vivo electroporation to knock down NeuroD1 in the olfactory bulb neurogenic system. For validation, three different NeuroD1 specific shRNA vectors were cotransfected with a NeuroD1 expression construct into COS-7 cells. Western blot analysis demonstrated that two of the shRNAs, sh775 and sh776, efficiently inhibited production of the NeuroD1 protein, whereas sh777 induced a less efficient down-regulation (sh775, 94.6%; sh776, 96.9%; sh777, 78.4%; corrected for loading against α tubulin; Fig. 4*A*). All three shRNAs were used for further in vivo studies.

The NeuroD1 shRNA vectors or an empty control were coelectroporated with either a nuclear ds-red or a GFP expression plasmid, which allowed visualization of transfected cells. Analyses were performed at 4 and 15 dpe. At 4 dpe, the repartition of transfected cells in the VZ/SVZ, with strongly positive cells in the VZ and weaker labeled cells in the SVZ, was indistinguishable among animals electroporated with the different control and knockdown vectors as exemplified for sh775 (Fig. 4*B* and *C*). Furthermore, equivalent amounts of cells entered the RMS (Fig. 4*D* and *E*), and the overall distribution of cells along the entire rostro-caudal axis was comparable under the different conditions (Fig. 4*F*). This was expected because NeuroD1 is not expressed as long as cells are present in the SVZ and RMS (Fig. 1).

We demonstrated before that, at 15 dpe, GFP-expressing control neurons were generally correctly integrated in the OB (25). In agreement, under control conditions, GFP-positive cells were found predominantly in the GCL or GL. Most of the cells in the GL showed complex neuronal morphologies and extended multiple processes into one or more glomeruli (class 1; Fig. 4*G* and *H*). Dendrites of GFP-positive cells in class 1 were covered with dendritic spines, indicating synaptic integration into the glomerular circuitry (Fig. 4*H*). Cells with single, nonbranched processes (class 2) or cells lacking processes entirely (class 3) were rare at this time point (Fig. 4*G*).

When the two highly active NeuroD1-specific shRNAs sh775 and sh776 were electroporated, the vast majority of cells in the GL showed simple morphologies with few or no processes (classes 2 and 3), whereas cells with complex neuronal morphologies were sparse (Fig. 4*G*). When the less-efficient shRNA sh777 was expressed, an intermediate degree of neuronal maturation was observed (Fig. 4*G*), suggesting a dose-dependent action of

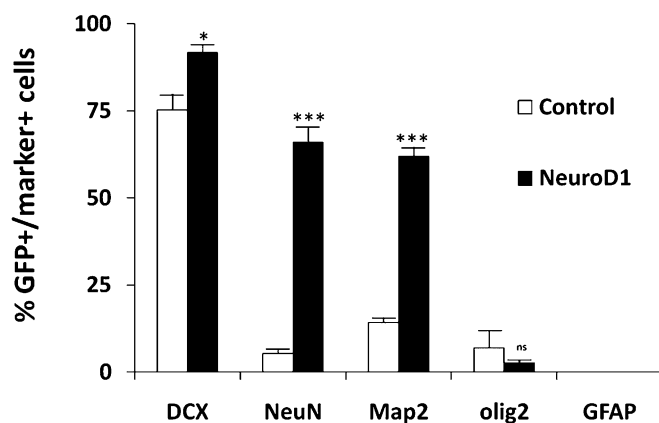


Fig. 3. NeuroD1 induces generic neuronal markers in vivo. Molecular phenotype of the cells located in the periventricular region (level 4 in Fig. 2*I*). Quantification representing the percentage of GFP-positive cells expressing the respective markers. DCX: control, $75.2 \pm 4.5\%$, $n = 5$; NeuroD1, $91.7 \pm 2.2\%$, $n = 5$. NeuN: control, $5.2 \pm 1.4\%$, $n = 8$; NeuroD1, $65.9 \pm 4.5\%$, $n = 9$. Map2: control, $14.1 \pm 1.4\%$, $n = 3$; NeuroD1, $61.9 \pm 2.7\%$, $n = 3$. Olig2: control, $6.8 \pm 5\%$, $n = 3$; NeuroD1, $2.5 \pm 0.5\%$, $n = 3$. GFAP: control, 0% , $n = 3$; NeuroD1, 0% , $n = 2$. Errors bars indicate SEM. Statistics: DCX and Map2, unpaired *t* test; NeuN, Mann-Whitney test. ns, not significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

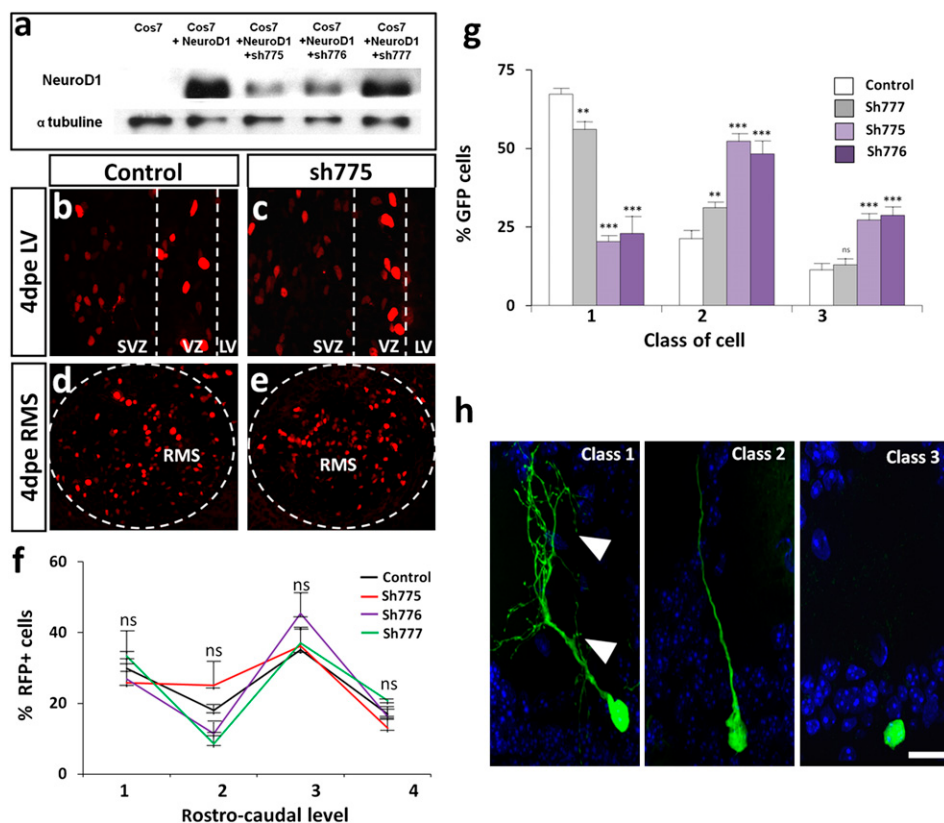


Fig. 4. In vivo terminal neuronal differentiation of PGC is impaired in absence of NeuroD1. (A) Western blot analysis of protein extracts from cos-7 cells transfected with NeuroD1 or in combination with different NeuroD1 specific shRNAs. sh775 and sh776 strongly repressed NeuroD1 protein expression (94.6% and 96.9%, respectively), whereas sh777 repressed NeuroD1 by 74.8%. (B–E) Consequences of loss-of-function of NeuroD1 via in vivo postnatal electroporation at 4 and 15 dpe. (B–E) No differences were observed at the level of the lateral ventricle or in the RMS at 4 dpe. (F) Cell distribution along the rostro-caudal axis was normal (definition of levels in Fig. 2). (G and H) Consequences of NeuroD1 knockdown on PGN morphology at 15 dpe. (G) Whereas shRNAs showing a strong effect on NeuroD1 expression strongly inhibited morphological differentiation, the weakly active shRNA 777 had only a minor effect compared with control. (H) Examples of cells that served for classification of PGN. Class 1 cells present primary and secondary branching. Dendritic spines (arrowheads) indicate their synaptic integration in OB circuitry. Class 2 cells present a single primary branch. Class 3 cells present a spherical morphology and no branching. Errors bars indicate SEM. Statistics, unpaired *t* test. ns, not significant. ***P* < 0.01; ****P* < 0.005. (Scale bar: 100 μm in B–E; 20 μm in H).

NeuroD1 under these conditions. Comparable results were obtained for the GCL. As in the PGL, knockdown of NeuroD1 induced a dose-dependent inhibition of terminal neuronal differentiation (Fig. S9A and B).

Thus, knockdown of NeuroD1 did not notably interfere with early steps of interneuron generation, but induced a specific defect in the acquisition of the differentiated neuronal phenotype in the OB.

Discussion

Although considerable information is available concerning the generation, specification, and migration of neurons, little is known concerning the factors and regulatory cascades that maintain the immature neuronal precursor status or induce the exit from this state and trigger terminal differentiation. Using a systematic approach, we identified NeuroD1 as a candidate for the latter function and validated this role using gain- and loss-of function approaches.

In *Xenopus*, a late function of NeuroD1 has been suggested based on two lines of evidence (13). First, NeuroD1 is transiently expressed in territories where neuronal differentiation occurs. Second, misexpression of NeuroD1 causes the premature differentiation of neuronal precursors into neurons. However, the observation that NeuroD1 could also convert presumptive epidermal cells into neurons pointed toward a determination function. Therefore, a doubtless discrimination between a proneural and a terminal differentiation function was not possible.

The above-cited pioneering work in the frog has been extended through the analysis of mice with mutations in the NeuroD1 gene (14, 15, 21). In the hippocampal dentate gyrus of such animals, granule cell precursors are generated correctly in the neuroepithelium and invade the hippocampal anlage. However, in the target structure, precursors show a severe deficit in proliferation, and a defined dentate gyrus is not formed (15). In the mutant cerebellum, generation and migration of early precursors appear not to be affected. Nevertheless, once these cells become postmitotic, massive cell death

is observed and the cerebellum is severely affected (14). Thus, in these systems a late function of NeuroD1 is already suggested. However, because of the complexity of the models and the relatively low level of resolution, the available information is still fragmentary.

We attempted to clarify the role of NeuroD1 in neuronal differentiation by analyzing its function during olfactory neurogenesis. Using SAGE, microarray, in situ hybridization, and lacZ knockin into the NeuroD1 locus, we have demonstrated that NeuroD1 is expressed in mature neurons of the OB but is absent from immature stages. These findings are in contrast to recent expression data based on a NeuroD1 antibody, suggesting expression of the transcription factor already in the SVZ and RMS (23, 29). However, our loss-of-function approach based on RNAi shows that NeuroD1 is dispensable for generation and migration of precursors but is necessary for their transition into neurons in the target layer. These findings are in agreement with those of a recent study based on conditional NeuroD1 mutants, which showed a comparable defect in the OB (29).

In addition, we have shown that ectopic expression of NeuroD1 is sufficient to induce instant and widespread neuronal differentiation in the periventricular area, a region that is specialized in the maintenance of stem cells (4, 5, 30). Overexpression of the determination factor Pax6 did not induce a comparable phenotype, demonstrating the specificity of the observed phenotype. In agreement with a function of NeuroD1 in terminal differentiation in the absence of a specification function, we found that NeuroD1-induced ectopic neurons in vivo express generic neuronal markers such as NeuN and Map2 but fail to express specific markers for subtypes of PGN, except for a very small proportion (<5%) that shows low levels of immunoreactivity for calretinin. It has been recently demonstrated that stem cells at the level of the lateral ventricle are already specified to generate defined neuronal subtypes in the OB (6, 7). However, the minor effect that we show here suggests that, despite the predisposition of these cells, important elements for complete subtype specification are lacking. For

example, coincident presence of Pax6 and Dlx2 is necessary for the specification of dopaminergic neurons in GL. However, coexpression of both factors is found only in the RMS and not in the periventricular region (9, 12). Thus, it appears that neuronal cells in the olfactory system have to pass through subsequent maturation steps, during which they acquire the competence (for example, by the generation of the correct transcription factor context) to express the full spectrum of neuronal characteristics. Such a notion is further supported by findings in the cortex, where NeuroD1 is expressed after Pax6 and Tbr2 but before Tbr1 and thus in a defined transcription factor context.

NeuroD1 induced neurons survive in their ectopic positions for ~1 week but disappear over the following days until no living cells can be detected. Neuronal survival is dependent on trophic support and proper integration into the circuitry (31). NeuroD1-induced neurons are found in an environment that is suited for the maintenance, proliferation, and migration of progenitors, but probably not for synaptogenesis. Alternatively, it appears conceivable that NeuroD1 expression needs to be transitory and switched off after terminal differentiation is induced. Indeed, not all cells in the GL do express NeuroD1 at a given time point. This suggests either that only a subpopulation of PGN expresses the transcription factor or that all cells express NeuroD1 during a defined time window. Furthermore, in *Xenopus* and in several structures of the developing mouse brain, NeuroD1 expression is transitory (13, 32, 33). In our gain-of-function experiments, using the actin promoter, we did not allow dynamic expression of the protein under its natural regulatory elements, which, however, might be crucial for complete differentiation.

This work demonstrates that expression of a single transcription factor can induce massive ectopic neuronal differentiation of neural stem cells in the vertebrate forebrain. The existence of postnatal and adult neurogenesis holds potential for the treatment of neuro-

degenerative diseases (34). However, in many experimental paradigms, transplanted or recruited cells fail to undergo differentiation into neurons and either transdifferentiate into glia or remain immature precursors (18, 35). It appears conceivable to combine such approaches with the strong neuronal differentiation inducing activity of NeuroD1.

Materials and Methods

Animals. Animals were treated according to guidelines approved by the French ethics committee.

Microdissection and Magnetic Activated Cell Sorting. Microdissection and magnetic activated cell sorting (MACS) are described in detail elsewhere. Microarray analysis was performed using Agilent (Agilent) microarrays. Expression plasmids used in this study were based on an eukaryotic expression vector based on the chicken β -actin promoter and the CMV enhancer (pCX-mcs2). For shRNA knockdown, U6 expression plasmids were obtained from Sigma-Aldrich. Cultured primary cells from the periventricular region of P1 mice were transfected using Amaxa reagents. All details on in vivo electroporation were previously published (25). Antisense RNA probes for in situ hybridization were generated using the Dig-RNA labeling kit (Roche) (36).

Quantification and Statistical Analysis Data are presented as mean $\pm$ SEM. The designation n represents the number of animals analyzed except in Fig. 3M, where n represents the number of cells analyzed. Student's unpaired t test or Mann-Whitney test were used to assess differences between data groups using Instat software (Graph Pad Software). Differences were considered statistically significant when $P < 0.5$.

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