Mesencephalic origin of the rostral Substantia nigra pars reticulata

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Abstract In embryonic development, the neurons that will constitute a heterogeneous nucleus may have distinct origins. The different components of these populations reach their final location by radial and tangential migrations. The Substantia nigra pars reticulata (SNR) presents a high level of neuronal heterogeneity. It is composed by GABAergic neurons located in the mes-diencephalic basal plate. These inhibitory neurons usually display tangential migrations and it has been already described that the caudal SNR is colonized tangentially from rhombomere 1. Our aim is to unveil the origin of the rostral SNR. We have localized a Nkx6.2 positive ventricular domain located in the alar midbrain. Nkx6.2 derivatives’ fate map analysis showed mainly a rostral colonization of this GABAergic neuronal population. We confirmed the mesencephalic origin by the expression of Six3. Both transcription factors are sequentially expressed along the differentiation of these neurons. We demonstrated the origin of the rostral SNR; our data allowed us to postulate that this nucleus is composed by two neuronal populations distributed in opposite gradients with different origins, one from rhombomere 1, caudal to rostral, and the other from the midbrain, rostral to caudal. We can conclude that the SNR has multiple origins and follows complex mechanisms of specification and migration. Our results support vital information for the study of genetic modifications in these extremely complex processes that result in devastating behavioral alterations and predisposition to psychiatric diseases. Understanding the development, molecular identity and functional characteristics of these diverse neuronal populations might lead to better diagnosis and treatment of several forms of neurological and psychiatric disease.

Keywords Substantia nigra pars reticulata · Tangential migration · GABAergic neurons · Nkx6.2 · Six3

Introduction

The Substantia nigra (SN) is a complex nucleus located not only in the mesencephalic basomedial territory (Puelles 2007; Moreno-Bravo et al. 2012; Puelles et al. 2012) but it is also extended along the pretectum, thalamus and pre-thalamus (diencephalic prosomeres). It is divided into a pial superficial part, SN pars reticulata (SNR), constituted by GABAergic neurons (GABAn) and a more internal SN pars compacta (SNC), primarily containing dopaminergic neurons (Hanaway et al. 1970). There is, nonetheless, some intermixing of dopamine neurons within the SNR (González-Hernández and Rodríguez 2000). The dopaminergic neurons are a deeply studied population due to their implication in several motor syndromes such as Parkinson’s disease; however, the molecular diversity and regulation of GABAn development are only beginning to be understood. These GABAn control several aspects of behavior, play important roles in psychiatric diseases, susceptibility to drugs of abuse and are also important targets for several medical treatments for these diseases.
(Jhou et al. 2009; Vargas-Perez et al. 2009; Cohen et al. 2012).

The SNR and the internal segment of the globus pallidus provide the major output projections of the basal ganglia system where the final stage of information processing takes place. These cell groups are mainly composed of GABAergic neurons; they integrate inputs from all other components of the basal ganglia system (striatum, globus pallidus, subthalamic nucleus) and elaborate the message sent by this system to extrinsic structures (Rinvik et al. 1976). For this purpose, SNR neurons project to the superior colliculus, reticular formation and thalamus, mainly to the ventral lateral and ventral anterior region. SNR GABAergic neurons also issue local axon collaterals that carry out an important role of inhibition within the SNC itself. Therefore, the SNR constitutes one of the main output pathways of the basal ganglia system regulating mainly voluntary movements (Beckstead et al. 1979).

The mechanisms of GABAergic development in the midbrain have been, surprisingly, neglected until recently. On the one hand, Nakatani et al. (2007) studied the spatial patterning relevant to the GABAergic neurogenesis. Seven distinct progenitor domains were identified along the midbrain neuroepithelium dorsoventral axis (m1–m7; Nakatani et al. 2007; renamed in Puelles et al. 2012). GABAergic neurons are originated from the domains m3 to m5 (corresponding to alar ventrolateral, basal lateral and basal intermediate domains; Puelles et al. 2012), and later in development also from m1 and m2 (corresponding to alar dorsal and alar lateral domains).

On the other hand, Achim et al. (2012) analyzed molecular regulation of ventral tegmental area (VTA) and SNR GABAergic differentiation. They demonstrated that GABAergic neurons of these regions, mainly the caudal portion, were originated in rhombomere 1 (r1) and occupied their final destination by tangential migration. Nevertheless, the origin of the main rostral mes-diencephalic SNR GABAergic population was not described.

Our previous data pointed out that Nkx6.2 transcription factor plays an important role in the determination and differentiation of the mesencephalon and diencephalon ventral neuronal populations. We found a Nkx6.2 dynamic expression pattern in the developing mes-diencephalic basal plate, with an early alar positive ventricular domain. However, later in development, only the pre-Edinger-Westphal remains Nkx6.2 positive (preEW; described previously as Interstitial mesencephalic nucleus by Moreno-Bravo et al. 2010). In other regions of the brain, Nkx6.2 positive ventricular territories give rise to a massive amount of derivatives which switch off its expression as they differentiate and migrate tangentially (Fogarty et al. 2007). This study prompted us to analyze the fate of the mesencephalic Nkx6.2 derivatives. We found out that they contribute to several basal populations, being the SNR among them. With the aim to verify their mesencephalic origin, we selected Six3, a positive marker of SNR (Conte et al. 2005). This transcription factor belongs to the sine oculis family (Oliver et al. 1995) and it already has been involved in GABAergic development (Virolainen et al. 2012). It has a complex expression pattern restricted to the foreand midbrain (Conte et al. 2005). Summarizing, our working hypothesis postulates a complex multiple origin of the SNR neurons. We demonstrate, using the transcription factors Nkx6.2 and Six3, the mesencephalic neuronal contribution to the SNR. The GABAergic generated in the Nkx6.2 positive ventricular domain populated the SNR in a rostrocaudal gradient.

Results

Nkx6.2 alar derivatives contribute to SNR

To study the behavior of Nkx6.2 GABAergic derivatives in the midbrain and diencephalon, we used the Nkx6.2tmcre/+; tdTomatoflx/+; Gad65gfp/+ transgenic mouse. In these mice, all the neurons that were generated from Nkx6.2 positive progenitors were labeled in red fluorescent color, the GABAergic in green fluorescent color and the Nkx6.2 derived GABAergic neurons in yellow fluorescent color. First, we analyzed along embryonic development the contribution of Nkx6.2 derivatives to the SNR. Our data allowed us to determine the time window of this process between E8.5 and E10.5. In E18.5 embryos induced by tamoxifen at E8.5, we detected a small number of Nkx6.2 GABAergic in the SNR (Fig. 1a). In embryos induced at E9.5, we found a huge increment in the number of double positive neurons (Fig. 1b). The induction one day later showed only some scattered double positive neurons (Fig. 1c). Therefore, we demonstrated that the peak of Nkx6.2 contribution to the SNR takes place at E9.5. These data were corroborated by the analysis of long-pulse BrdU labeling at E9.5 (Achim et al. 2012). The proliferating neuroblasts at E9.5 were fated to become SNR GABAergic neurons (Fig. 1d). We used immunohistochemistry for tyrosine hydroxylase to detect the dopaminergic neurons of the SNC and confirm the location of the green fluorescent protein (GFP) positive GABAergic in the SNR (Fig. 1e).

The positive Nkx6.2 neuroblasts switch off its expression as they differentiate and migrate into the mantle layer. The use of the Nkx6.2tmcre/+ allowed us to label permanently the Nkx6.2 derivatives (note the difference between Nkx6.2 expression and Nkx6.2 derivatives at E11.5, bracket in Fig. 1f, g) and, therefore, to analyze their contribution to the different neuronal populations. We followed the behavior of Nkx6.2 derivatives, labeled at E9.5, along...
development. At E13.5, we observed a dense positive group of cells in the mantle layer (Fig. 1h). The positive neurons that contribute to the preEW (Puelles et al. 2012) appeared in the basomedial territory. At E15.5, we found the mesencephalic reticular formation (mRt) highly colonized by Nkx6.2 derivatives. We identified the SNR by the superficial location of the red fluorescent protein (RFP) positive neurons; the preEW appeared now clearly defined (Fig. 1i). Finally, at E18.5, the three territories were clearly identified. The SNR displayed a dense group of RFP-positive neurons. The Nkx6.2 derivatives in the mRt showed the typical net-like organization of this complex population. The preEW appeared also densely colonized (Fig. 1j). As previously described, in the basal mesencephalic area, only the preEW contains neurons that retain the Nkx6.2 expression (Moreno-Bravo et al. 2010).

These results demonstrate that SNR is partially originated from a Nkx6.2 positive ventricular territory. In addition, we have clearly shown that at E9.5 there is a peak of proliferation and determination of SNR neurons originated in this Nkx6.2 positive territory.

Rostrocaudal distribution of Nkx6.2 derivatives

The contribution of r1 GABAn to the SNR displays a clear asymmetric distribution along the rostrocaudal axis, being

**Fig. 1** Selected mesencephalic transversal paraffin section through Nkx6.2tmcre/+; tdTomato<sup>flox/+</sup>; Gad67gfp/+ embryos. a–c E18.5 sections immunoreacted against α-RFP in red (Nkx6.2 derivatives) and α-GFP in green (GABAn). Tamoxifen induced at E8.5, E9.5 and E10.5, respectively. d E18.5 section labeled against α-BrdU in red and α-GFP in green. e E18.5 section immunoreacted against α-TH in red and α-GAD67 in green. f, g E11.5 induced at E9.5 sections hybridized with Nkx6.2 probe and immunoreacted against α-RFP, respectively. The bracket labels the Nkx6.2 positive ventricular domain. The dotted line indicates the area of SNR. preEW pre-Edinger-Westphal, mRt mesencephalic reticular formation, SNC Substantia nigra pars compacta, SNR Substantia nigra pars reticulata. Scale bars 250 μm in a–e and h–j; 100 μm in f, g.
more abundant in the caudal SNR and almost absent in the rostral part (Achim et al. 2012). We analyzed the distribution of the Nkx6.2 derivatives along this rostrocaudal axis. In mes-diencephalic transversal sections of a Nkx6.2\textsuperscript{tmcre/+}; tdTomato\textsuperscript{flox/+}; Gad67\textsuperscript{gfp/+} E18.5 embryo induced at E9.5, we detected a gradual distribution of Nkx6.2 derivatives along the SNR. In rostral sections, we observed a high number of double-labeled neurons tightly packed in the area of the SNR (Fig. 2a–c). Therefore, the GABAergic Nkx6.2 derivatives are clearly more abundant in the rostral than in the caudal portion of the nucleus (Fig. 2a–f). We quantify this phenomenon selecting a fixed area in rostral and caudal sections of the SNR. The proportion of double-labeled neurons against the total number of GABA\textsuperscript{gfp} displayed a distribution that clearly proved that the observed phenomenon is statistically significant (Fig. 2g).

Undoubtedly, the SNR is not a homogeneous population. In fact, we have proven that there are clear rostrocaudal differences in the origin of this neuronal nucleus as it was previously suggested (Achim et al. 2012). This diversity in origin could account for functional differences of the SNR neurons (discussed below).

Tangential migration of SNR subpopulation

The alar location of the Nkx6.2 positive ventricle and the SNR basal situation forced us to study the existence of a tangential migration process. The Nkx6.2 derivatives must migrate in a rostro-ventral direction to colonize mainly the rostral portion of the SNR (Fig. 3a). Therefore, they not only cross the alar–basal boundary but several interprosomic limits. We can summarize, in a schematic horizontal section to the diencephalon, the final location of the Nkx6.2 derivatives. First, close to the ventricle, the tangentially migrated preEW neurons; second, close to the pial surface, the tangentially migrated SNR neurons and finally, in the mantle layer, the radially migrated mRt neurons (Fig. 3a').

First, we analyzed the behavior of the Nkx6.2 derivatives through the SNR development using the Nkx6.2\textsuperscript{tmcre/+}; tdTomato\textsuperscript{flox/+}; Gad67\textsuperscript{gfp/+} strain. At E13.5, the preEW is already well developed. It is located close to the ventricle and distributed along the tegument of the diencephalic prosomeres (Fig. 3b). In the mantle layer of the alar midbrain, we localized a compacted group of GABAergic Nkx6.2 derivatives (Fig. 3b). These double-labeled neurons will later on migrate to the SNR final location. At E15.5, this compacted group is already positioned close to the pial surface and it already started the colonization of the mid-

![Fig. 2](image_url)
**diencephalic basal plate (Fig. 3c).** The GABAergic Nkx6.2 derivatives occupied their final destination at E18.5. They are located close to the pial surface of the basal plate (Fig. 3d). The mRt revealed its complexity with Nkx6.2 and non-Nkx6.2 derivatives, GABAergic and non-GABAergic neurons and its distribution along alar and basal domains (Fig. 3d).

Second, we carried out an in vitro time-lapse experiment with an E13.5 horizontal section of a Nkx6.2tmcre/+; tdTomato+/-; Gad67gfp/+ embryo at E13.5 (b), E15.5 (c) and E18.5 (d) immunoreacted against α-RFP in red (Nkx6.2 derivatives) and α-GFP in green (GABAn). Static images from a time-lapse experiment where a horizontal section with their endogenous fluorescence was recorded at 0 h (e), 8 h (f) and 16 h (g). The white dotted lines indicate the alar–basal boundary. The arrows show the tangential migration of SNR Nkx6.2-GABAergic derivatives. a/b alar–basal boundary, Mb midbrain, mRt mesencephalic reticular formation, Hb hindbrain, PHy peduncular hypothalamus, preEW pre-Edinger-Westphal, p1 pretectum, p2 thalamus, p3 prethalamus, SNR Substantia nigra pars reticulata, THy terminal hypothalamus; Scale bars 100 μm in a–c; 80 μm in d–f.

**Fig. 3** Tangential migration. a, a′ Schematic diagrams of a late stage neural tube (lateral view) and a horizontal slice displaying the tangential migration of Nkx6.2 derivatives. The line indicates the section plane. The black dotted lines indicate the boundary of neuromeres. The blue dotted line indicates the alar–basal boundary. The arrows show the tangential migration of the Nkx6.2 derivatives. Vibratome horizontal sections through Nkx6.2tmcre/+; tdTomato+/-; Gad67gfp/+ embryos at E13.5 (b), E15.5 (c) and E18.5 (d) immuno-reacted against α-RFP in red (Nkx6.2 derivatives) and α-GFP in green (GABAn). Static images from a time-lapse experiment where a horizontal section with their endogenous fluorescence was recorded at 0 h (e), 8 h (f) and 16 h (g). The white dotted lines indicate the alar–basal boundary. The arrows show the tangential migration of SNR Nkx6.2-GABAergic derivatives. a/b alar–basal boundary, Mb midbrain, mRt mesencephalic reticular formation, Hb hindbrain, PHy peduncular hypothalamus, preEW pre-Edinger-Westphal, p1 pretectum, p2 thalamus, p3 prethalamus, SNR Substantia nigra pars reticulata, THy terminal hypothalamus; Scale bars 100 μm in a–c; 80 μm in d–f.

Six3 expression in the SNR

The analysis of the data obtained suggested a mesencephalic origin of the rostral SNR. The expression of Nkx6.2 caudal to the isthmic constriction prompted us to search for a transcription factor that could serve as a selective marker of the mesencephalic Nkx6.2 derivatives (Moreno-Bravo et al. 2012). We decided to use the transcription factor Six3, as it already described its expression in the SNR (Conte et al. 2005). It displays a complex expression pattern in the fore- and midbrain and it is never expressed in the hindbrain. In an E18.5, Six3 expression displayed a sharp boundary between the midbrain and the isthmus (Fig. 4a). This expression coincided with the well-known Otx2 caudal expression limit (Fig. 4b) that precisely points out the location of the midbrain–hindbrain boundary. In transversal sections to an E12.5 midbrain, Six3 is expressed in two domains located in the mantle layer. The wider domain is located in the alar plate and the thinner one in the basal plate (arrow and arrowhead, respectively, in Fig. 4c). The Pax6 positive territory in the basal intermediate region separates both domains (Fig. 4d, d′). The location of the Six3 positive neurons coincided with the location of the Nkx6.2 derivatives (compare Fig. 1g with Fig. 4c).

We analyzed Six3 mesencephalic expression along embryonic development. At E12.5, we could detect the two domains. Some positive neurons could be found in the superficial mantle layer of the dorsal domain (Fig. 5a). At E14.5, scattered positive neurons were localized in the mantle layer. A group of positive neurons was localized close to the pial surface (arrow in Fig. 5b). At E18.5, the Six3 positive neurons were localized in their final location.
destination. In the basal medial territory, the Dark-schewitsch nucleus was strongly positive for \( \text{Six}3 \) (Fig. 5c). A packed group of \( \text{Six}3 \) positive neurons was located in the presumptive area of the SNR (arrow in Fig. 5c).

At this last stage, we studied the co-localization of \( \text{Six}3 \) with several markers. First, we verified that \( \text{Six}3 \) positive cells are present in the SNR region constituted by \( \text{Nkx}6.2 \) derivatives (Fig. 5d). We confirmed that SNR \( \text{Nkx}6.2 \) derivatives also co-expressed \( \text{Six}3 \) performing a double immunohistochemistry (Fig. 5e–g). The SNR \( \text{RFP} \)-positive neurons co-expressed \( \text{Six}3 \) (arrows in Fig. 5g). Second, we confirmed that these \( \text{Six}3 \) positive neurons were GABAergic components of the SNR via GAD67 immunoreactivity (arrow in Fig. 5h). Simultaneously, we corroborated the Dk nucleus identification, as it is a compacted GABAergic basal population (Fig. 5h). Finally, we corroborated through tyrosine hydroxylase immunoreactivity the location of the SNC to prove the correct recognition of the SNR (arrow in Fig. 5i).

Hence, \( \text{Six}3 \) is expressed in SNR GABAn \( \text{Nkx}6.2 \) derivatives. All these data together, allowed us to confirm that the SNR is partially colonized by GABAn originated in the mesencephalic \( \text{Nkx}6.2 \) positive ventricular domain.

**Discussion**

The SN GABAn origin is still not completely unveiled despite the studies developed in the last years. Here, we planned to find out the origin of the rostral SNR GABAn. We demonstrated that the rostral SNR is colonized by alar mesencephalic \( \text{Nkx}6.2 \) derivatives. These tangentially migrated neurons populate mainly the rostral diencephalic part of the SNR, but are also present in the caudal mesencephalic part.

We hypothesized that the rostral SNR had an alar mesencephalic origin. There were several preliminary data in the literature that supported our hypothesis. Fate map analysis of the mesencephalic basal plate \( \text{Shh} \) positive derivatives demonstrated that the SNR is derived from a \( \text{Shh} \) negative territory (Joksimovic et al. 2009; Achim et al. 2012). Therefore, the generation of the SNR GABAn in the \( \text{Shh} \) negative r1 basal plate or in the mesencephalic alar plate appeared as plausible hypothesis. It has been also proven that the SNR GABAn are partially derived from r1 (Achim et al. 2012). These authors do not exclude a mesencephalic or diencephalic origin of the rostral SNR as the rhombomeric originated.
GABAn described concentrate in the caudal SNR (Achim et al. 2012).

Our previous studies of Nkx6.2 expression pattern showed that this transcription factor has a ventricular positive domain adjacent to the alar–basal boundary. This domain gives rise to the preEW, a neuronal population that maintains Nkx6.2 expression and migrates tangentially into the basal plate. Nkx6.2 derived neurons usually display tangential migration events. The analysis of the cortical GABA interneurons origin demonstrated that the Nkx6.2 positive ventricular domain in the medial ganglionic eminence gives rise to a huge amount of GABAn that switch off the expression of the gene as they differentiate and migrate into the cortex (Fogarty et al. 2007). This result supported our hypothesis that the Nkx6.2 positive ventricular domain could contribute neurons to different mesencephalic populations, by tangential migration, as proposed by Verney et al. 2001.

The use of a transgenic mouse line (Nkx6.2<sup>tmcre/</sup>; tdTomato<sup>fluox/</sup>; Gad67<sup>gfp/</sup>) allowed us to label all the derivatives generated from Nkx6.2 positive neuroblasts (RFP+) and also to distinguish the GABAn (GFP+) among them. This analysis demonstrated the colonization of basal neuronal structures by these derivatives. The SNR was among these neuronal populations. We found RFP+ neurons distributed in a rostrocaudal gradient along the SNR. This distribution was opposed and complementary to the r1 derived GABAn described by Achim et al. (2012).

**Fig. 5** Midbrain Six3 expression pattern. Selected transversal parafin sections through embryos at different stages. Six3 in situ hybridization at E12.5 (a), in E14.5 (b) and in E18.5 (c). The arrows in b and c indicate the area of SNR. d E18.5 Six3 in situ hybridization in blue combined with α-RFP in brown, the arrow indicates the SNR. e, f E18.5 immunofluorescent reacted against α-RFP in red (Nkx6.2 derivatives) and α-SIX3 in green. g Combined e and f, showing the coexpression between Nkx6.2 derivatives and positives Six3 neurons. g' A high magnification of g to better illustrate the colabelling, indicated by arrows. Cells expressing Six3 and Nkx6.2 contribute to the SNR. h, i α-GAD67 and α-TH in brown, respectively. The arrows in h, i indicate the SNR. Dk Darkschewitsch nucleus, SNC Substantia nigra pars compacta, SNR Substantia nigra pars reticulata. Scale bars 100 μm in a, b, g; 200 μm in e–i.
The distribution of these two subpopulations is translated in neuronal morphological differences, the rostral lateral SNR is populated by fusiform GABAergic with major cellular diameter and the caudomedial SNR by elongated GABAergic with minor diameter (González-Hernández and Rodríguez 2000). These differences have been also illustrated by SNR projections labeling. Both territories project to the same thalamic areas but the rostral SNR also projects to the centrolateral and thalamic reticular nucleus (Gulcebi et al. 2012).

Due to the proximity of the territories involved (r1, isthmus and midbrain), we confirmed the mesencephalic origin of the RFP+; GFP+ neurons using Six3 as specific mesencephalic marker. It reported its expression in the SNR (Conte et al. 2005) and it is never expressed along development in the hindbrain (Oliver et al. 1995). Therefore, taking into account of all these data, we can postulate that the SNR GABAergic are originated, at least, from two different sources, r1 and midbrain. The GABAergic originated in these two territories are distributed in two opposite rostrocaudal gradients and certainly present neuronal morphological, projections and functional differences.

The molecular regulation of the GABAergic differentiation associated with the populations described is distinct from the rest of mesencephalic GABAergic (Lahti et al. 2013). In the Gata2cko mutant, all the midbrain GABAergic populations were transformed to a glutamatergic phenotype, except for the SNR and mRF (Lahti et al. 2013). This information together with our data allowed us to postulate that Nkx6.2 and Six3 must participate sequentially in the genetic cascade responsible of rostral SNR and mRF neuronal differentiation program.

It has been described that the GABAergic development in the different regions of the central nervous system is regulated by diverse genetic mechanisms. Transcription factors, such as Ascl1, Helt or Gata2, have been shown to be selectively required for the development of midbrain GABAergic. However, GABAergic associated with the dopaminergic nuclei in the VTA and SN do not require any of them (Peltopuro et al. 2010). Indeed, as they develop independently of the known transcriptional regulators, the VTA and SN GABAergic differentiation is absent specifically in the BL domain of the midbrain (location of the Nkx6.2+ ventricular domain; Achim et al. 2013). As expected, the generation of the SNR is strongly affected.

All this data support the hypothesis that Tal2 regulates the differentiation of the SNR GABAergic. This regulation takes place in the BL mesencephalic domain where Nkx6.2 is expressed in the ventricular neuroblasts and Six3 is expressed in the early-differentiated neurons in the mantle layer. These early GABAergic migrate tangentially until their final destination in the SNR.

Another important conclusion to highlight from our data is that we have identified an alar ventricular domain in the mesencephalon able to give rise to different neuronal types. Early in development, it produces glutamatergic neurons that tangentially colonize the preEW nucleus. Later, the Nkx6.2 positive neuroblasts switch and generate GABAergic that tangentially and radially populate the SNR and mRF, respectively.

Finally, the midbrain dopaminergic neurons (SNC and VTA) and their development have been under intensive research due to their relation to Parkinson’s disease. However, importance of the VTA- and SN-associated GABAergic for the activity of dopaminergic pathways and behavioral control has become increasingly evident (Vargas-Perez et al. 2009). In fact, GABAergic in the ventral mesodiencephalic region are highly important for the function of dopaminergic pathways that regulate multiple aspects of behavior and movement control. These complex morphological and functional structures display intricate developmental processes with multiple origins and migratory routes. Consequently, all our results contribute to implement our knowledge of how these important GABAergic populations are generated.

Material and methods

Mouse strains

The mouse lines used and their genotyping have been described previously: Nkx6.2 cre ER T2 (Feil et al. 1997;
Sousa et al. 2009), GAD67-GFP (Tamamaki et al. 2003), R26R-CAG-tdTomato, obtained from Jackson Laboratories (strain 007905). A loxP-flanked STOP cassette prevents transcription of the downstream RFP variant (tdTomato) in the TdTomato reporter mice.

Nkx6.2<sup>Cre/+;</sup> tdTomato<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>; are generated by crossing homozygous mouse males (Nkx6.2<sup>cre/cre</sup>) with homozygous reporter females (tdTomato<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>). The triple mutant embryos, Nkx6.2<sup>Cre/+;</sup> tdTomato<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>; Gad67<sup>gfp/+</sup> were generated by crossing homozygous mouse males (Nkx6.2<sup>cre/cre</sup>) with double heterozygous females (tdTomato<sup>fl<sup>ox</sup>/fl<sup>ox</sup></sup>; Gad67<sup>gfp/+</sup>). For tamoxifen induction, we administer 4 mg of tamoxifen (Sigma, T-5648) (20 mg/ml dissolved in corn oil, Sigma C-8267) per 30 g of pregnant mouse with a gavage needle.

For staging, the day of vaginal plug was counted as embryonic day 0.5 (E0.5). For immunohistochemistry and in situ hybridization, embryos were fixed in 4% paraformaldehyde in PBS overnight and completely dehydrated for storage at −20 °C. Samples were paraffin embedded and sectioned at 7 μm or agarose embedded (1%) and sectioned at 150 μm.

All mouse experiments were performed according to protocols approved by the Universidad Miguel Hernandez OEP committee.

Immunohistochemistry and in situ hybridization

IHC was performed as described (Moreno-Bravo et al. 2014). The following antibodies were used: Rabbit α-RFP IgG (MBL Cat. No. PM005; 1:100), Mouse α-GAD67 IgG (Millipore Cat. No. MAB5406; 1:300), Rabbit α-TH IgG (Institute Jacques Boy Cat. No. 268020234; 1:1000), Rabbit α-PAX2 IgG (Zymed 71-6000; 1:5), Sheep α-BrdU IgG (Abcam ab1893; 1:150), Guinea pig α-SIX3 IgG (Rockland 200-201-A26; 1:200.)

In situ hybridization analyses on paraffin sections were performed as previously described (Moreno-Bravo et al. 2014) using digoxigenin-labeled RNA probes. Mouse cDNA probes used for in situ hybridization analysis were Six3 (P. Gruss), Gad67 (W. Wurst), Nkx6.2 and Otx2 (A. Simeone).

Birth dating by BrdU labeling

For detection of the peak of neurogenic proliferation, BrdU was administered intraperitoneally to the pregnant females (3 mg/100 g body weight) every 2 h, for a period of 10 h (five injections in total) starting at desired stages.

Time lapse

For the time-lapse experiments, the embryos were extracted and dissected in cold PBS. Samples were embedded in low melting point agarose (4%) and sectioned at 250 μm. The sections were collected using Krebs 1X medium (Krebs, glucose, NaHCO<sub>3</sub>, Hapes 1 M 1%, penicillin/streptomycin 1%, Gentamicina 0.2%) at 4 °C. The selected slice was placed in a polycarbonate membrane (MilliCell PIMORG50) with neurobasal medium and incubated during the experiment (37 °C, 5% CO<sub>2</sub>), and sectioned at 250 μm.

For confocal imaging, a Leica SPE-II DM5550 laser scanning confocal microscope was used. A TCS-SP2-AOBS laser scanning spectral inverted confocal microscope (fitted with temperature and CO<sub>2</sub> control; Leica Microsystems) was used for live imaging of brain slice culture. Images were collected every 20 min during 16 h. All the focal planes were merged to visualize the maximum projection. Videos were processed with Imaris and ImageJ software.

Microscopy and quantification

IHC and ISH staining on paraffin and vibratome sections were visualized under fluorescence automated DM6000B microscope and MZ16FA Fluorescence Stereomicroscope (for wide-field microscopy), running Leica Application Suite (LAS) AF6000 Software (version 2.0.2), equipped with a DFC350-FX (monochrome) or DC500 (color) digital cameras. Images were processed and assembled with Adobe Photoshop software.

For quantification, cells were counted only from the rostrocaudal SNR domain. A fix area (275 μm x 687.5 μm) in this region was used to count GABA and Nkx6.2 positive neurons and then compare rostrocaudal SNR axis. A standard Student’s t test was used for comparing the mean values of the data sets.

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