Development/Plasticity/Repair

Origin and Molecular Specification of Globus Pallidus Neurons

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The mechanisms controlling the assembly of brain nuclei are poorly understood. In the forebrain, it is typically assumed that the formation of nuclei follows a similar sequence of events that in the cortex. In this structure, projection neurons are generated sequentially from common progenitor cells and migrate radially to reach their final destination, whereas interneurons are generated remotely and arrive to the cortex through tangential migration. Using the globus pallidus as a model to study the formation of forebrain nuclei, we found that the development of this basal ganglia structure involves the generation of several distinct classes of projection neurons from relatively distant progenitor pools, which then assemble together through tangential migration. Our results thus suggest that tangential migration in the forebrain is not limited to interneurons, as previously thought, but also involves projection neurons and reveal that the assembly of forebrain nuclei is more complex than previously anticipated.

Introduction

The vertebrate CNS is organized in two main types of anatomical and functional units, named brain nuclei and laminated structures. These two types of building blocks use different histological arrangements to group neurons with specific patterns of connections and therefore play a fundamental role in organizing neural circuitries in the brain. Development of brain nuclei and laminar structures requires the coordination of two major histogenetic processes, neurogenesis and neuronal migration, which, respectively, account for the generation of different neuronal classes and their subsequent allocation in the CNS. Over the past decades, we have learned a great deal about the mechanisms controlling these processes during the formation of laminar structures, most notably through the analysis of the cerebral cortex (Gupta et al., 2002; Rakic, 2007). In contrast, our knowledge on the mechanisms that generate discrete brain nuclei, with the appropriate size, location, and neuronal composition, is still very limited.

The generation of neuronal diversity in the cerebral cortex is mainly achieved by the sequential production of cell types in a defined order. Thus, cortical progenitors go through a series of changes in intrinsic properties that control their competence to make different classes of projection neurons with very diverse connectivity (Molyneaux et al., 2007; Leone et al., 2008). This process is coordinated with the translocation of newborn cells by radial migration, in which cortical neurons use the long processes of radial glial cells as a "guiding cable" that they follow to migrate to their final position (Rakic, 1972; Rakic et al., 1974; Nadarajah and Parnavelas, 2002; Noctor et al., 2004). Neuronal diversity in the cerebral cortex also increases with the incorporation of interneurons born at remote locations and that reach the cortex through tangential migration (Corbin et al., 2001; Marín and Rubenstein, 2001; Wonders and Anderson, 2006).

It has long been assumed that the formation of nuclei in the forebrain involves a similar sequence of events than in the cerebral cortex, although the resulting neuronal assembly has a very different organization. Development of the striatum, for example, is thought to involve three sequential steps: (1) production of different classes of projection neurons from the same common progenitor pool in the lateral ganglionic eminence (LGE); (2) radial migration of striatal projection neurons to form a distinct neuronal assemble in the telencephalic mantle; and (3) tangential migration of interneurons from a remote progenitor pool in the medial ganglionic eminence (MGE), which further increases cell diversity within the nucleus (Deacon et al., 1994; Olsson et al., 1995, 1998; Marín et al., 2000; Wichterle et al., 2001; Hamasaki et al., 2003). A similar process has been proposed recently for the generation of some nuclei in the amygdala (Hirata et al., 2009).
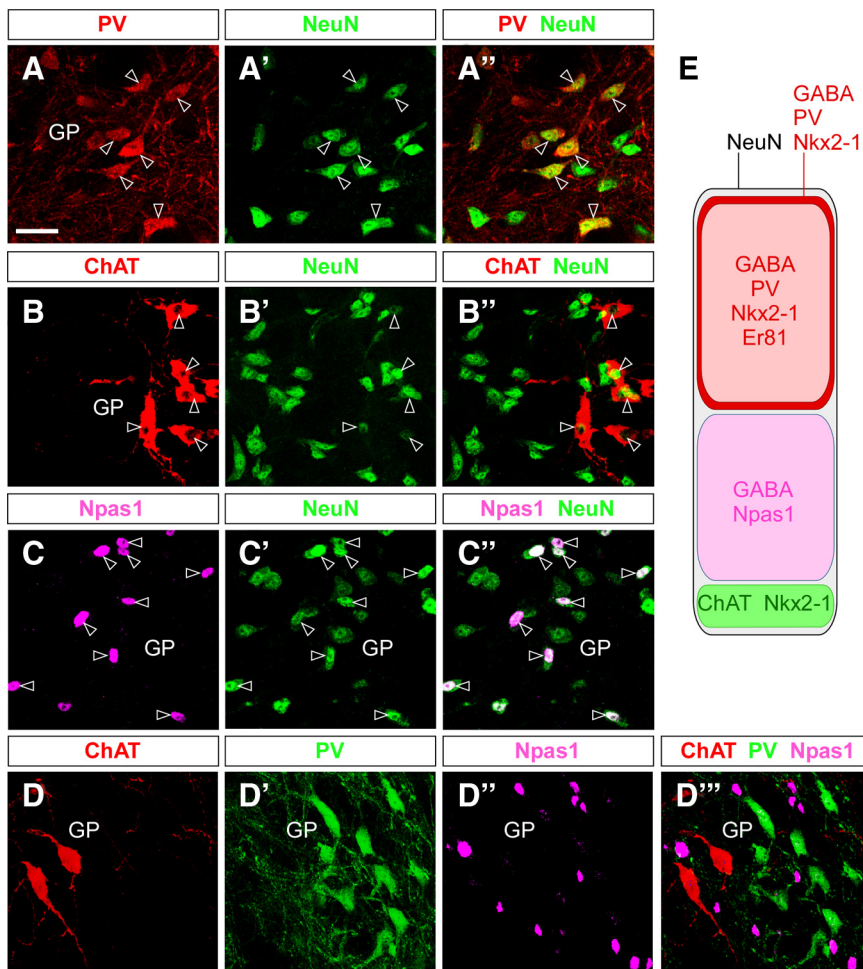
Figure 1. Neuronal populations in the globus pallidus. *A–D'''*, Coronal sections through the telencephalon of P60 wild-type mice showing coexpression of PV (*A, A'', D', D'''*), ChAT (*B, B'', D, D'''*), Npas1 (*C, C'', D'', D'''*), and the neuronal marker NeuN (*A', A'', B', B'', C', C''*) in the GP. Open arrowheads point to examples of double-labeled cells. Note that PV, ChAT, and Npas1 are expressed by primarily nonoverlapping populations of neurons in the GP. *E*, Schematic representation of the three distinct neuronal populations present in the GP and the molecular markers used to identify them. Scale bar, 50 μm.

To determine whether brain nuclei in the forebrain are formed following the general principles outlined above, we investigated the development of another basal forebrain structure, the globus pallidus (GP). This important node basal ganglia nucleus contains several distinct classes of projection neurons but few interneurons (Kita, 1994; Kita and Kitai, 1994; Nambu and Llinás, 1994, 1997; Bevan et al., 1998; Cooper and Stanford, 2000; Kita and Kita, 2001; Kita, 2007). Thus, the GP represents an ideal model to understand the origin of projection neuron diversity and their relationship to the different modes of migration during the assembly of nuclear structures. Our results indicate that the assembly of the GP involves at least five different progenitor lineages from three distinct embryonic domains.

Materials and Methods

Mouse lines. Wild-type and green fluorescent protein (GFP)-expressing transgenic mice (Hadjantonakis et al., 2002) were maintained in a CD1 background. *Nkx2-1–Cre* (Xu et al., 2008), *Dbx1–Cre* (Bielle et al., 2005), *Rosa–EYFP* (Srinivas et al., 2001), and *Pax6–Cre* transgenic mice were maintained in a mixed C57BL/6 × 129/SvJ × CBA background. The bacterial artificial chromosome (BAC) used to generate *Pax6–Cre* mice spanned ~181 kb of genomic DNA around the *Pax6* locus and was purchased from BacPac Resources Center (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). The codon-improved Cre re-

combinase with a nuclear localization signal was fused to the translation initiation codon using an approach based on the PCR and followed by an simian virus 40 polyadenylation signal. The line was produced by pronuclei microinjection at the Institut Clinique de la Souris (Illkirch, France). The day of vaginal plug was considered as embryonic day 0.5 (E0.5). Animals were kept at the Instituto de Neurociencias, and experiments were performed following Spanish and European Union regulations.

In utero *electroporation.* Timed-pregnant *Nkx2-1–Cre* or wild-type females were deeply anesthetized, and the abdominal cavity was cut open. Embryos were exposed in the uterus, and 1 μl of a 1 μg/μl solution of *pCAGGS–LoxP–STOP–LoxP–EGFP* [MGE and preoptic area (POA) electroporations] or *pCAGGS–EGFP* or *pCAGGS–RFP* (LGE electroporations) plasmids were injected into the lateral ventricle of the telencephalon through the uterine wall. Square electric pulses of 35 V and 50 ms were passed through the uterus five times, spaced 950 ms apart, using a square pulse electroporator (CUY21E; Nepa GENE). DNA was specifically directed toward the subpallium, as described previously (Borrell et al., 2005).

In utero *transplantation.* In utero ultrasound-guided transplantation of MGE-derived cells was performed as described previously (Pla et al., 2006). For each experiment, the MGE from 8–10 E12.5 GFP-expressing mice were dissected under a stereomicroscope. Explants were washed in 0.5 ml of L-15 medium (Invitrogen) containing DNase I (100 μg/ml), and cells were mechanically dissociated by repeated pipetting. Dissociated cells were pelleted by centrifugation (5 min, 1000 rpm), resuspended in 6 μl of L-15 medium with DNase I, and kept on ice until injection. All donor pregnant females were injected with bromodeoxyuridine (BrdU) (50 mg/kg) 12 h before dissection. High-density cell suspensions (25,000 cells/μl) were front loaded into beveled glass micropipettes (50 μm diameter) prefilled with mineral oil and mounted in a pressure microinjector (VisualSonics). Recipient pregnant females at E12.5 were anesthetized with sodium pentobarbital (0.625 mg/10 g, i.p.), and their uterine horns were exposed and mounted under an ultrasound microscope (VisualSonics). The tip of the micropipette was inserted into the MGE under real-time ultrasound guidance, and 100–150 nl of cell suspension was injected.

In situ *hybridization and immunohistochemistry.* For *in situ* hybridization, embryonic brains were fixed overnight in 4% paraformaldehyde (PFA) and embedded in Tissue-Tek OCT compound (Sakura Finetek Europe). Twenty micrometer frozen coronal and horizontal sections were hybridized with digoxigenin-labeled probes against *Er81* (Lin et al., 1998), as described previously (Flames et al., 2007). For immunohistochemistry, embryonic brains were fixed in 4% PFA at 4°C from 2–6 h. Brains were then embedded and cut frozen at 20 μm in the cryostat or sectioned in the vibratome at 60 μm. Postnatal mice were anesthetized with an overdose of sodium pentobarbital and transcardially perfused with 4% PFA. Postnatal brains were removed, fixed for 1–3 h at 4°C, and cryoprotected in 30% sucrose in PBS. Brains were then cut frozen on a sliding microtome at 40 μm. The following primary antibodies were used: rat anti-BrdU (1:100; Accurate Chemical and Scientific), goat anti-choline acetyltransferase (ChAT) (1:100; Millipore Bioscience Research Reagents), rabbit anti-Er81 (1:5000; a gift from T. Jessell, Columbia University, New York, NY), rabbit anti-GABA (1:1000; Sigma), chicken anti-GFP (1:3000; Aves Labs), rabbit anti-Nkx2-1 (1:2000; Biopat), rabbit

anti-Npas1 (1:1000; a gift from S. McKnight, University of Texas Southwestern, Dallas, TX), mouse anti-neuronal nuclei (NeuN) (1:500; Millipore Bioscience Research Reagents), rabbit anti-parvalbumin (PV) (1:5000; Swant), and mouse anti-PV (1:5000; Swant). The following secondary antibodies were used: goat anti-chicken 488, donkey anti-rabbit 555, donkey anti-mouse 555 (all from Invitrogen), and donkey anti-rat cyanine 3 (Jackson ImmunoResearch). The immunofluorescence detection of enhanced yellow fluorescent protein (EYFP) was performed using an anti-GFP antibody. 4',6'-Diamidino-2-phenylindole (Sigma) was used for fluorescent nuclear counterstaining. GP neurons were mapped and quantified using a microscope equipped with Neurolucida software.

Quantification. For the birthdating of GP neurons, CD1 wild-type pregnant females were injected with BrdU (50 mg/kg) at E10.5, E11.5, E12.5, and E13.5. PV/BrdU and Npas1/BrdU double-labeled cells were counted using images obtained in a confocal microscope from three different rostrocaudal levels of the GP from three different animals per stage. For the quantification of colocalization patterns, labeled cells were counted from three different sections throughout the rostrocaudal extent of the GP from three different animals per stage. In all cases, data were expressed as average ± SEM.

Results

Molecular profiling of globus pallidus neurons

Previous studies have shown that the GP mostly consists of GABAergic projection neurons, some of which express the calcium binding protein PV (Fig. 1*A*–*A''*) (Kita and Kita, 2001). In addition, the GP contains some cholinergic neurons (ChAT) (Fig. 1*B*–*B''*), although they do not seem to constitute a major component of this structure. Instead, these cholinergic neurons represent an extension of the basal magnocellular complex that intercalates through the medial aspect of the caudoputamen nucleus and the GP (Kita, 2007). As part of a large effort to delineate distinct molecular profiles in the mouse subpallium, we found that the adult GP also contains many cells that express Npas1 (Fig. 1*C*), a basic helix–loop–helix/PAS domain transcription factor that is expressed by many GABAergic neurons in the forebrain (Zhou et al., 1997; Cobos et al., 2006). We observed that virtually all Npas1-expressing cells in the GP are neurons, as identified with antibodies against the neuronal marker NeuN (Fig. 1*C*–*C''*). Analysis of the relative contribution of these populations to the neuronal complement of the GP suggests that they may represent nonoverlapping groups (PV, 44 ± 6%; ChAT, 9 ± 1%; Npas1, 33 ± 5%; averages ± SEM; n = 3). Indeed, triple immunohistochemical analysis revealed that PV-, ChAT-, and Npas1-expressing neurons constitute three distinct neuronal populations in this region (Fig. 1*D,E*). Because virtually all neurons in the GP are either GABAergic or cholinergic (data not shown), these experiments also indirectly indicated that Npas1-expressing neurons in the GP are GABAergic.
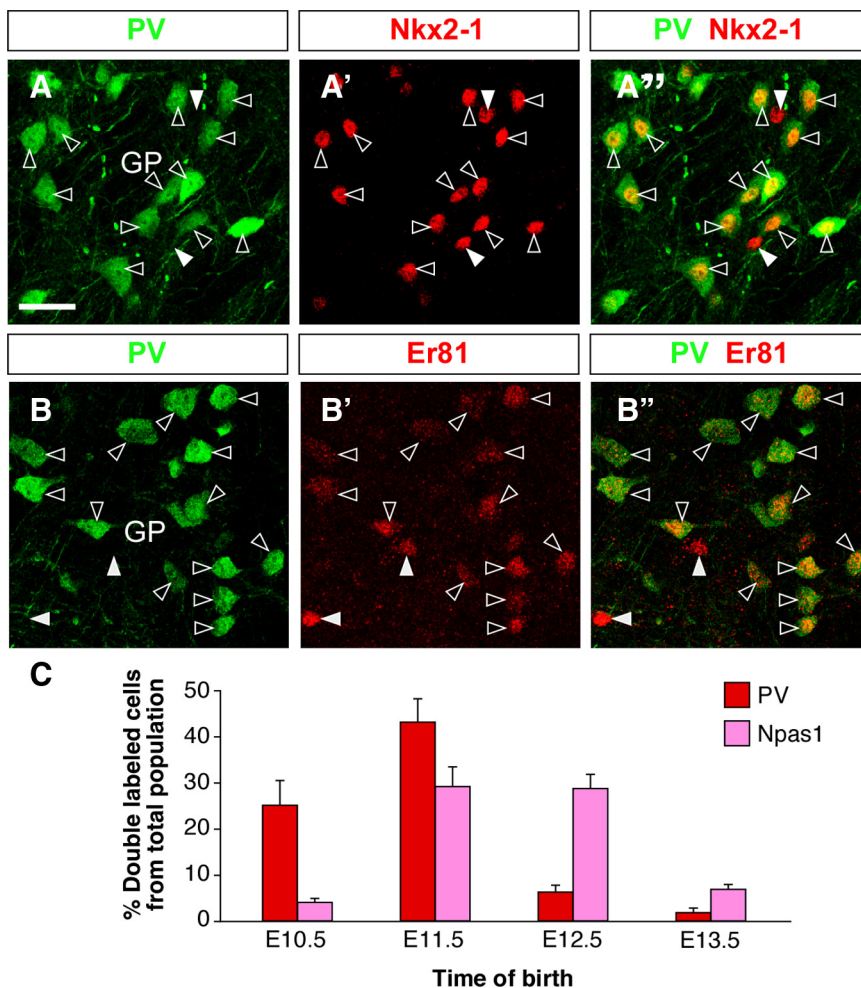

Figure 2. Molecular profile and time of neurogenesis distinguish the two main classes of GABAergic neurons in the globus pallidus. *A*–*B''*, Coronal sections through the telencephalon of P60 wild-type mice showing coexpression of PV (*A*, *B*) with Nkx2-1 (*A'*, *A''*) and Er81 (*B'*, *B''*) in the GP. Open arrowheads point to examples of double-labeled cells, whereas filled arrowheads point to neurons that express only one of the markers. *C*, Quantification of the percentage of PV-expressing and Npas1-expressing GP neurons labeled with BrdU at different developmental stages. Histograms show average ± SEM. E10.5, 24.91 ± 5.34% (PV), 3.88 ± 0.09% (Npas1); E11.5, 42.91 ± 5.14% (PV), 29.23 ± 3.79% (Npas1); E12.5, 6.13 ± 1.61% (PV), 28.72 ± 3.00% (Npas1); E13.5, 1.59 ± 0.29% (PV), 6.65 ± 0.86% (Npas1). Scale bar, 50 μm.

We analyzed the expression of other transcription factors in the adult GP. We have shown previously that Nkx2-1 is expressed in virtually all progenitor cells in the MGE and the POA, as well as in postmitotic cells that populate the GP and other subpallial structures (Sussel et al., 1999; Marín et al., 2000; Flames et al., 2007; Nóbrega-Pereira et al., 2008). Analysis of the expression of Nkx2-1 in the adult GP revealed that virtually all PV-positive (PV^{+}) neurons also contain this transcription factor (Fig. 2*A*–*A''*). Moreover, most PV^{+} neurons were found to also express the ETS transcription factor Er81 (88 ± 6%; n = 2) (Fig. 2*B*–*B''*). Because PV[+] and ChAT^{+} neurons in the GP accounted for most Nkx2-1-expressing neurons in this structure (data not shown), these experiments also suggested that pallidal Npas1-expressing neurons do not contain Nkx2-1. In summary, our analysis suggested that the mouse GP contains two major populations of GABAergic neurons: Nkx2-1/PV/Er81 neurons and Npas1 neurons (Fig. 1*E*).

We next investigated the timing of generation for the two populations of GABAergic neurons present in the GP. To this end, we performed BrdU birthdating at four embryonic days,
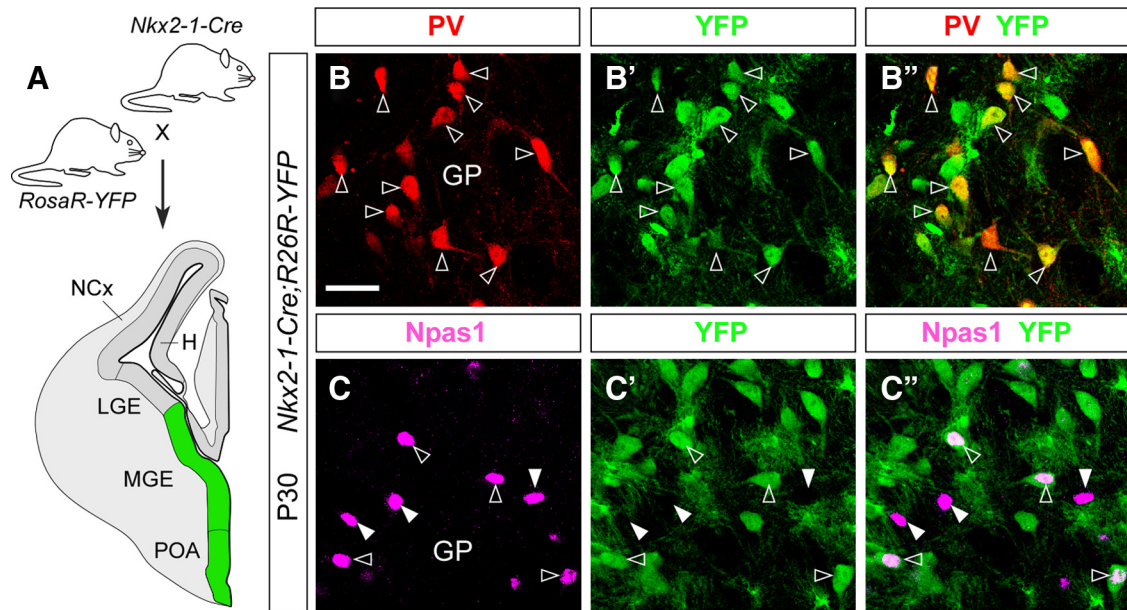
Figure 3. Fate-mapping analysis of the globus pallidus with *Nkx2-1–Cre* mice. **A,** Schematic of the experimental design, indicating the progenitor domain labeled in *Nkx2-1–Cre* mice. **B–C″,** Coronal sections through the telencephalon of P30 *Nkx2-1–Cre;Rosa26R–YFP* mice showing coexpression of PV (**B, B″**) and Npas1 (**C, C″**) with YFP (**B′, B″, C′, C″**) in the GP. Open arrowheads point to examples of double-labeled cells, whereas filled arrowheads point to neurons that express only one of the markers. H, Hippocampus; NCx, neocortex. Scale bar, 50 μm.

E10.5, E11.5, E12.5, and E13.5 and analyzed the distribution of labeled cells at postnatal day 14 (P14). Consistent with previous results in rats (Marchand and Lajoie, 1986), our results indicated that most GP neurons are generated at approximately E11.5 (Fig. 2*C*). We observed, however, important differences in the time of generation of the two main classes of GABAergic neurons in the GP. Thus, although most PV$^+$ neurons were produced between E10.5 and E11.5, neurons expressing Npas1 were generated through a protracted period of time, with a peak at approximately E12 (Fig. 2*C*). These results suggest that the dynamics of neurogenesis for GP neurons is more complex that previously recognized.

Nkx2-1–Cre fate mapping reveals multiple origins for globus pallidus neurons

A recent fate-mapping study using a transgenic mouse line in which the gene encoding for Cre recombinase is expressed under the control of the *Nkx2-1* gene (*Nkx2-1–Cre*) revealed that many PV-expressing neurons in the GP derive from Nkx2-1-expressing cells (Xu et al., 2008). To extend this study, we quantified the relative contribution of Nkx2-1 cells to the GABAergic neuronal complement in the GP (Fig. 3*A*). Analysis of *Nkx2-1–Cre; Rosa26R–YFP* mice at P30 revealed that virtually all PV-expressing neurons in the GP derive from Nkx2-1-expressing cells (99.1% ± 0.7%; *n* = 3) (Fig. 3*B–B″*). In contrast, only approximately half of the Npas1-expressing cells located in the GP seem to derive from an Nkx2-1-expressing territory (47.2 ± 2.3%; *n* = 3) (Fig. 3*C–C″*). These results suggest that many Npas1-expressing neurons arise from the MGE and/or POA, but they downregulate the expression of Nkx2-1 during embryonic stages. To test this idea, we analyzed the postmitotic expression of Nkx2-1 in the GP of *Nkx2-1–Cre;Rosa26R--YFP* mice at different embryonic stages. We found that a small percentage of Nkx2-1-derived cells in the GP do not express Nkx2-1 already at E13.5 (5 ± 1%; *n* = 2; data not shown), and this number increases at later embryonic stages (9 ± 2% at E15.5; *n* = 3; data not shown). These results are consistent with the idea that Nkx2-1-derived,

Npas1-expressing neurons in the GP downregulate the expression of Nkx2-1 as they mature. In summary, our results suggested that all PV-expressing neurons and approximately half of the Npas1-expressing GABAergic neurons in the GP derive from the MGE and/or the POA (Nkx2-1-expressing territories), whereas the remaining Npas1-expressing neurons originate from other regions.

The caudoventral region of the MGE is the origin of many globus pallidus neurons

To get more insight into the origin of GABAergic neurons in the GP, we next examined the development of those that derive from Nkx2-1-expressing territories. We failed to detect Npas1 expression in the basal ganglia during embryonic stages (data not shown), and so we could not monitor the migration of these cells from their place of origin. Similarly, GP neurons do not express detectable levels of PV until postnatal stages (Mitrofanis, 1992). In contrast, we found that probes against *Er81* label a population of cells emanating from the boundary between the caudal MGE and the POA during mid-embryonic stages, and many of these cells seem to invade the prospective territory for the developing GP (Fig. 4*A,B*). Because this transcription factor appears to be a reliable marker for PV-expressing neurons in the GP (Fig. 2*B– B″*), this observation suggested that GABAergic GP Nkx2-1/PV/ Er81-expressing neurons might derive from the caudoventral MGE (pMGE5 progenitor domain, as defined by Flames et al., 2007). To directly test this hypothesis, we performed a series of *in utero* electroporation experiments in which we targeted small domains of the MGE at E12.5, a stage in which a considerable proportion of GP neurons are generated (see Fig. 2*C*). To limit the electroporation target region to the *Nkx2-1*-expressing territories, we used a plasmid in which expression of *Gfp* was dependent on Cre-mediated recombination and used this vector to target the ventral telencephalon of E12.5 *Nkx2-1–Cre* transgenic mouse embryos (Fig. 4*C*). Analysis of mouse embryos at E15.5 revealed important differences depending on the electroporated region. We observed very few GFP-expressing cells in the GP
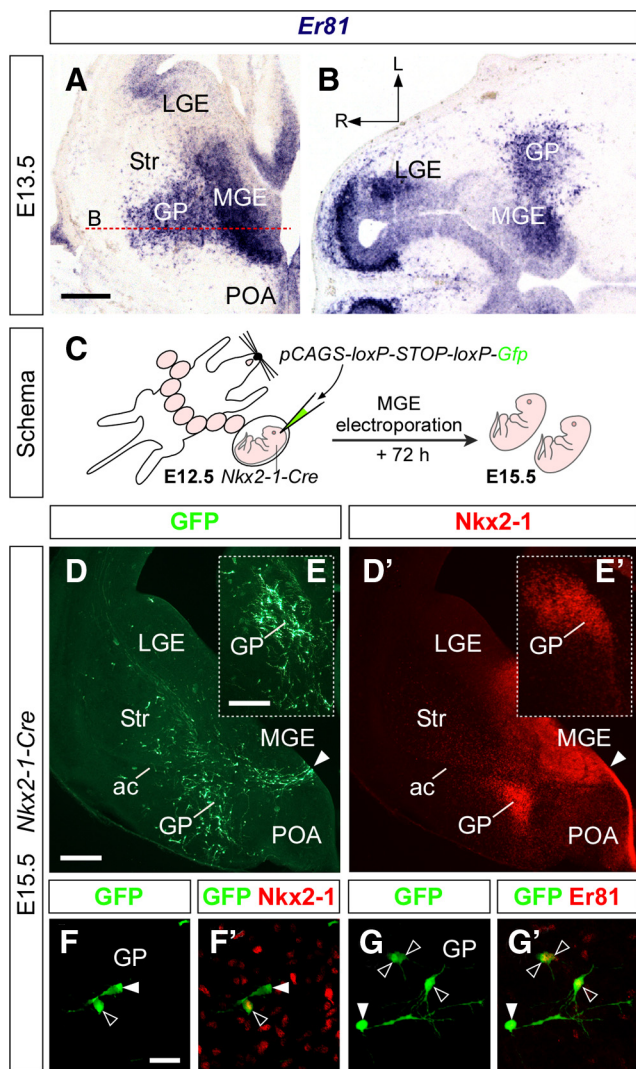
Figure 4. The MGE gives rise to cells that migrate to the embryonic globus pallidus. **A, B,** Coronal (**A**) and horizontal (**B**) sections through the telencephalon of E13.5 wild-type embryos showing the expression of *Er81* mRNA. The red dotted line in **A** indicates the plane of the section shown in **B**. The arrows indicate lateral (L) and rostral (R) orientations within the brain. **C,** Schematic of the experimental design. **D–E′,** A representative case of the distribution of GFP-expressing (**D, E**) and Nkx2-1-expressing (**D′, E′**) cells in a coronal section through the telencephalon of an E15.5 embryo in which the caudoventral MGE was electroporated at E12.5. The white arrowhead in **D** and **D′** indicates the place of electroporation. Note the presence of many GFP + cells in the ventral division of the GP (known as the ventral pallidum). **E** and **E′** are high-magnification images of the GP, in a section caudal to that shown in **D** and **D′** where this nucleus reaches its maximum area. **F–G′,** Images of representative cells found in the GP after electroporation in the caudoventral MGE. These cells typically stain for Nkx2-1 (**F′,** open arrowheads) and Er81 (**G′,** open arrowheads). However, some cells do not stain for either of the two markers (filled arrowheads). ac, Anterior commissure; Str, striatum. Scale bars: **A,** 200 μm; **D, D′,** 250 μm; **E, E′,** 100 μm; **F–G′,** 25 μm.

when intermediate and rostral levels of the MGE were specifically targeted (*n* = 4; data not shown). In contrast, targeting caudoventral regions of the MGE at E12.5 resulted in the accumulation of many GFP-expressing cells in the GP at E15.5 (*n* = 3 of 5 embryos) (Fig. 4*D,D′,E,E′*). Double immunohistochemistry revealed that, although most GFP + cells found in the GP also expressed Nkx2-1 and Er81, some cells did not (Fig. 4*F,F′,G,G′*). This suggests that the caudoventral MGE is a potential source for both populations of GABAergic GP neurons.

The MGE generates PV + and Npas1 + globus pallidus neurons

The previous experiments suggested that many neurons in the GP derive from the caudoventral aspect of the MGE. Because these experiments were analyzed at E15.5 to verify the electroporation sites and because many of the markers used to profile these cells are only expressed postmitotically, we could not characterized in detail this population of GP neurons. To this aim, we next performed long-term *in vivo* fate-mapping analysis of the MGE using *in utero* ultrasound-guided microtransplantation. In brief, we homotypically and isochronically transplanted E12.5 GFP-expressing cells from this region into wild-type host embryos and analyzed the molecular profile of neurons found in the GP of P14 transplanted mice (Fig. 5*A*). Transplanted MGE-derived cells populating the GP were found to express PV or Npas1 in similar proportions (Fig. 5*B,C*), suggesting that the MGE contributes to both populations of GABAergic cells in the GP. To ensure that MGE transplants did not include cells born in other regions that migrated through the MGE at the time of dissection, donor pregnant females were BrdU injected 12 h before dissection to identify transplanted cells that divided last in the donor environment. In these experiments, the relative contribution of GFP-expressing MGE-derived cells labeled with BrdU and PV or Npas1 was again similar (Fig. 5*D*), reinforcing the notion that the MGE contributes to both populations of GP cells. Finally, analysis of *in utero* ultrasound-guided microtransplantation experiments in which small fragments of the MGE were used as donor tissue (Flames et al., 2007) confirmed that GP neurons preferentially derive from ventral regions of the MGE (data not shown).

The POA generates globus pallidus neurons

The previous results indicated that GP cells are generated in the MGE. However, because *Nkx2-1* is also expressed by POA progenitors, it is possible that this region may also contribute to the neuronal complement of the GP. To begin to test this hypothesis, we performed a series of *in utero* electroporation experiments in which we specifically targeted the POA at E12.5 and analyzed the distribution of migrating neurons at E15.5 (Fig. 6*A*). We consistently found a few GFP-expressing cells in the GP in experiments in which the POA was targeted (*n* = 4 of 4 embryos) (Fig. 6*B–C′*). Double immunohistochemistry revealed that these cells also express Nkx2-1 (Fig. 6*D–D″*).

To extend these observations, we next performed fate-mapping studies using *Dbx1–Cre* mice (Bielle et al., 2005). In the developing telencephalon, some progenitor cells within the POA and its rostral extension in the septum (Bielle et al., 2005; Flames et al., 2007) express the transcription factor Dbx1. *Dbx1* is also expressed in the ventral pallium, but previous studies have shown that this region exclusively produces glutamatergic neurons (Bielle et al., 2005; Hirata et al., 2009). Thus, *Dbx1–Cre* mice seem useful to trace the contribution of POA/septal progenitors to GABAergic neurons in the GP (Fig. 6*E*). Analysis of *Dbx1–Cre; Rosa26R–YFP* mice at P30 revealed that a small fraction of PV-expressing neurons in the GP derive from *Dbx1*-expressing progenitors (6.7 ± 0.5%; *n* = 3) (Fig. 6*F–F″*). We also found that Dbx1-expressing progenitors gives rise to a small population of Npas1 + neurons in the GP (2 ± 0.2%; *n* = 3) (Fig. 6*G–G′*). These results demonstrated that the POA contributes to both PV + and Npas1 + neuronal populations in the GP.

The LGE gives rise to Npas1 + globus pallidus neurons

We next sought to determine the origin of Npas1 + neurons in the GP that do not derive from Nkx2-1-expressing territories. Anal-
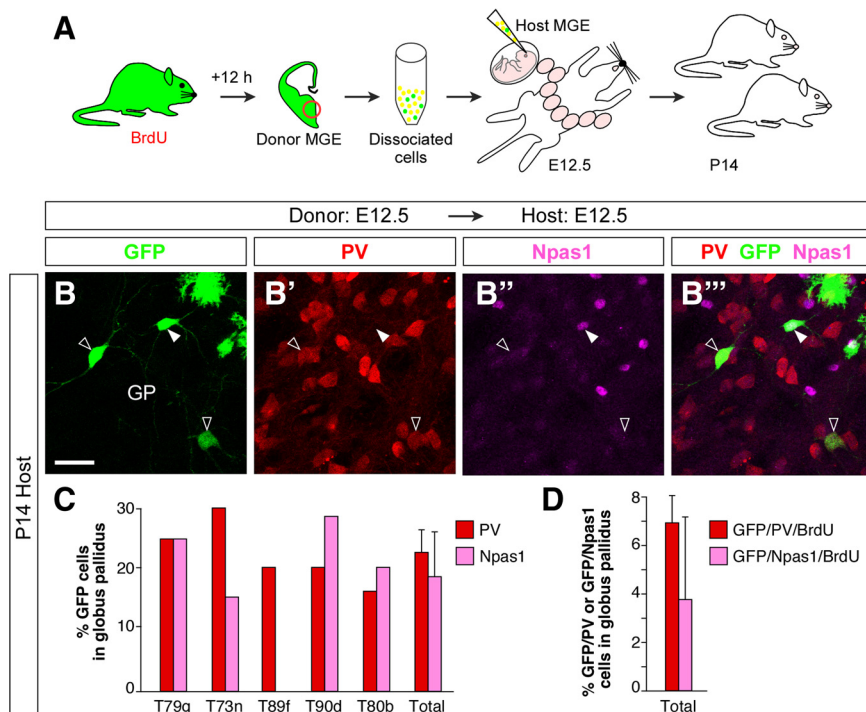
Figure 5. The MGE is the origin of globus pallidus neurons. **A**, Schematic of the experimental design. GFP $^+$ donor pregnant mice received a single injection of BrdU at E12.5. Twelve hours after BrdU injection, the MGE was dissected from embryos and dissociated. Donor MGE cells were then injected into the MGE of E12.5 host embryos, and host embryos were analyzed at P14. **B–B‴**, Coronal section through the GP of a transplanted P14 mouse showing the expression of PV (**B′**, **B‴**) and Npas1 (**B″**, **B‴**) in GFP $^+$ neurons derived from the MGE (**B**, **B‴**). Open arrowheads and filled arrowheads indicate PV/GFP and Npas1/GFP double-labeled cells, respectively. **C**, Quantification of the percentage of GFP $^+$ transplanted neurons expressing PV or Npas1 from five different experiments (T79g, T73n, T89f, T90d, T80b). Histograms show average $\pm$ SEM. T79g, 25.00 (PV), 25.00 (Npas1); T73n, 30.77 (PV), 15.38 (Npas1); T89f, 20.00 (PV); T90d, 20.51 (PV), 28.21 (Npas1); T80b, 16.00 (PV), 20.00 (Npas1); total, 22.46 $\pm$ 4.34 (PV), 17.72 $\pm$ 8.02 (Npas1). **D**, Quantification of the percentage of GFP $^+$ transplanted neurons expressing PV/BrdU or Npas1/BrdU in all experiments. Histograms show average $\pm$ SEM. Total: PV, 6.97 $\pm$ 1.15%; Npas1, 3.76 $\pm$ 3.56%. Scale bar, 50 μm.

but, because pallial cells do not seem to generate GABAergic cells for the subpallium (Chapouton et al., 1999; Stoykova et al., 2000; Toresson et al., 2000), we relied on these mice to study the contribution of the LGE to GABAergic cells in the GP (Fig. 7E).

Analysis of *Pax6–Cre;Rosa26R–YFP* embryos revealed mosaic GFP expression throughout the LGE and pallium (supplemental Fig. S2, available at www.jneurosci.org as supplemental material), indicative of a relatively low level of recombination (estimated at $\sim$20–25%) that was nevertheless confined within the expected domains of Pax6 expression. Analysis of these mice at P30 revealed that none of the PV $^+$ neurons in the GP stained with antibodies against GFP ($n = 3$) (Fig. 7F–F″). In contrast, many Npas1-expressing cells in the GP were found to derive from *Pax6*-expressing progenitors (23 $\pm$ 3%; $n = 3$) (Fig. 7G–G″).

Despite the mosaic recombination found in *Pax6–Cre* mice, which likely underestimated our observations, the previous results suggested that the GP Npas1-expressing cells that do not derive from the MGE or POA most likely derive from the LGE. If this were the case, we hypothesized that LGE-derived GP neurons should still be present in *Nkx2-1* mutant mice, in which a complete deletion of the GP was reported previously (Sussel et al., 1999). As described previously (Sussel et al., 1999), Nkx2-1-derived neurons were absent from the GP of *Nkx2-1* mutant embryos. This included cells expressing Lhx6 or Er81 (Fig. 8A,C and data not shown). In contrast, Npas1 $^+$ neurons were very abundant in a small structure located just medial to the developing striatum of *Nkx2-1* mutants, which resembled an atrophic GP (Fig. 8B,D). Indeed, we found that the density of Npas1 $^+$ neurons in the mutant GP doubled that found in controls ($n = 3$; control, 94.8 $\pm$ 7.4 cells/mm^2; *Nkx2-1$^{-/-}$*, 184 $\pm$ 9 cells/mm^2; $p < 0.05$, t test), probably because of the reduced volume occupied by this structure in *Nkx2-1* mutants. Altogether, these results strongly suggested that a large fraction of Npas1 $^+$ neurons in the GP derive from the LGE.

Discussion

Our results indicate that the assembly of forebrain nuclei is a much more complex process than previously anticipated. In contrast to the prevalent view that telencephalic nuclei primarily develop by radial migration of projection neurons from a single progenitor domain, our analysis indicates that assembly of a major forebrain nucleus, the GP, involves at least three major progenitor domains: the LGE, the MGE, and the POA (Fig. 9). Furthermore, because only one of these domains can be topologically radial to the developing GP (Misson et al., 1988) and this nucleus almost exclusively contains projection neurons (Kita, 2007), our results imply that tangential migration of projection neurons is required for the formation of forebrain nuclei. Thus, neuronal diversity within forebrain nuclei does not exclusively arise by the sequential generation of distinct classes of neurons

ysis of the expression of Npas1 in *Dlx5/6–Cre–IRES–Gfp* mice (Stenman et al., 2003), in which most subpallial GABAergic cells are labeled with GFP, revealed that virtually all Npas1 $^+$ neurons in the GP derived from Dlx5/6-expressing cells (data not shown). These results are consistent with the hypothesis that all Npas1 $^+$ neurons in the GP derive from the subpallium and suggest that the LGE might also contribute to this pallidal structure. To explore this possibility, we performed *in utero* electroporation experiments in which we targeted the LGE at E12.5 and analyzed the distribution of migrating neurons at E15.5 (Fig. 7A). Of note, in many of our experiments, we also labeled progenitor cells in the ventral pallium, although it is unlikely that pallial cells from this region give rise to GABAergic cells (Chapouton et al., 1999; Stoykova et al., 2000; Toresson et al., 2000). We found a few GFP-expressing cells in the GP in several experiments in which the LGE was targeted ($n = 3$ of 4 embryos) (Fig. 7B–C′). Double immunohistochemistry revealed that these cells do not express Nkx2-1 (Fig. 7D,D′).

To confirm these observations, we performed fate-mapping experiments. To this end, we made BAC transgenic mice expressing the recombinase *Cre* under the promoter region of the *Pax6* gene (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). In the absence of LGE-specific genes, we chose this transcription factor to fate-mapping LGE derivatives because *Pax6* transcripts are expressed in LGE progenitor cells during embryonic development (Sussel et al., 1999). *Pax6* is also strongly expressed by progenitor cells throughout the pallium
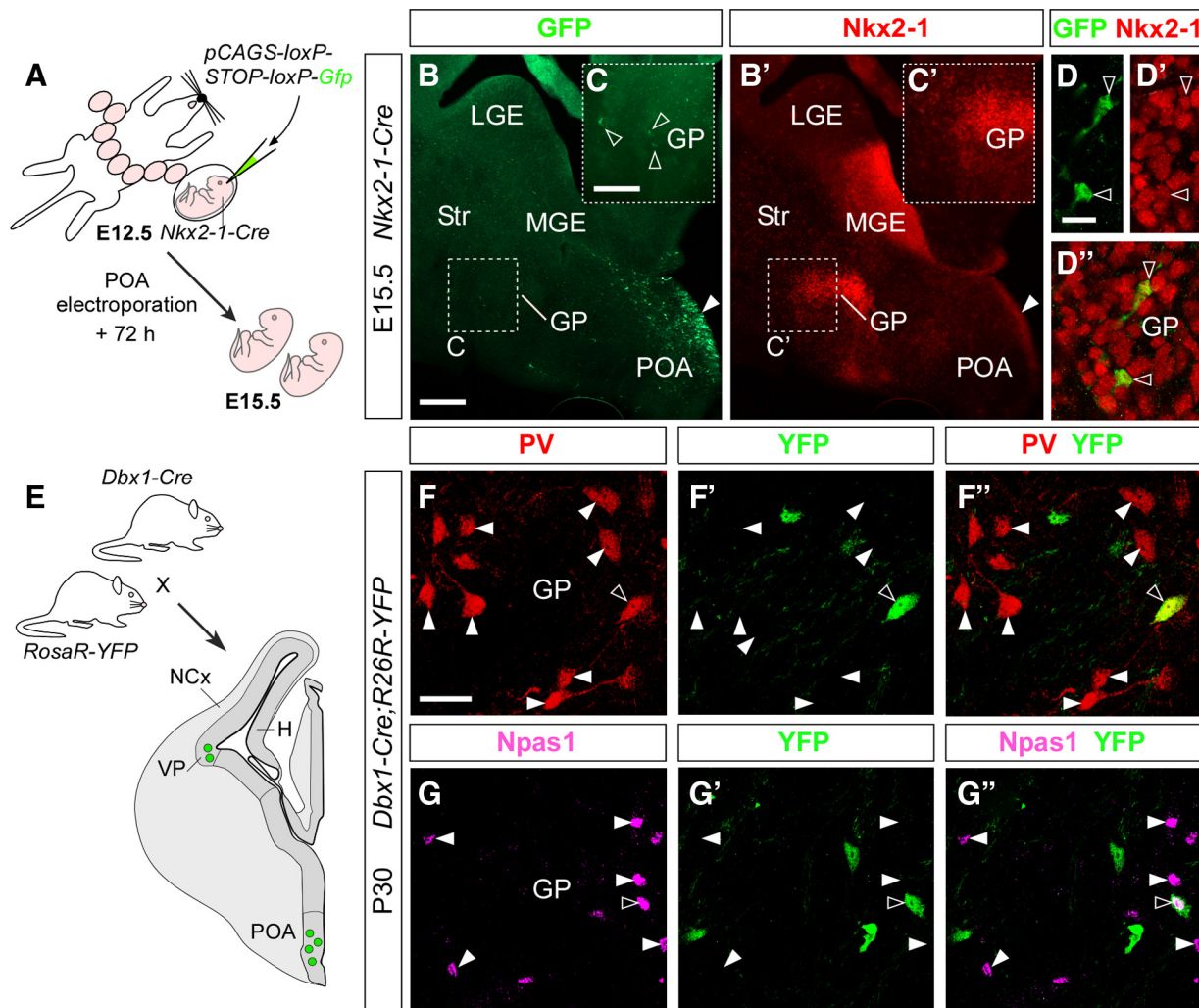
Figure 6. Origin of GP neurons in the POA. ***A***, Schematic of the experimental design. ***B–C′***, A representative case of the distribution of GFP-expressing (***B***, ***C***) and Nkx2-1-expressing (***B′***, ***C′***) cells in a coronal section through the telencephalon of an E15.5 embryo in which the POA was electroporated at E12.5. The white arrowhead in ***B*** and ***B′*** indicates the place of electroporation. ***C*** and ***C′*** are high-magnification images of the GP from the boxed area shown in ***B*** and ***B′***, respectively. ***D–D′***, Images of representative cells found in the GP after electroporation in the POA. ***E***, Schematic of the experimental design, indicating the progenitor domains labeled in *Dbx1–Cre* mice. Note that only a few progenitor cells within the ventral pallium (VP) and the POA express *Dbx1*. ***F–G″***, Coronal sections through the telencephalon of P30 *Dbx1–Cre;Rosa26R–YFP* mice showing coexpression of PV (***F***, ***F″***) and Npas1 (***G***, ***G″***) with YFP (***F′***, ***F″***, ***G′***, ***G″***) in the GP. Open arrowheads point to examples of double-labeled cells, whereas filled arrowheads point to neurons that express only one of the markers. Scale bars: ***B***, ***B′***, 250 μm; ***C***, ***C′***, 100 μm; ***D–D″***, 25 μm; ***F–G″***, 50 μm. Str, Striatum; H, hippocampus.

from a common progenitor but also through the tangential recruitment of distinct classes of neurons from multiple distant progenitor pools.

Multiple developmental origins underlie neuronal diversity in the globus pallidus

It has typically been assumed that neurons in the GP derive from the MGE (Smart, 1976; Marchand and Lajoie, 1986; Sussel et al., 1999), although direct evidence for this hypothesis is scarce (Pakzaban et al., 1993; Wichterle et al., 2001). Our *in utero* electroporation and transplantation experiments, along with the analysis of *Nkx2-1–Cre* mice, revealed that many GP neurons arise from the MGE. These results are consistent with previous genetic analyses in which deletion of genes expressed in the MGE, such as *Nkx2-1*, led to a prominent decrease in the number of GP neurons (Sussel et al., 1999). In contrast, previous *in utero* transplantation experiments did not identify the MGE as the main source of GP neurons in the mouse (Wichterle et al., 2001). The main difference between the experiments performed by Wichterle et

al. (2001) and our experiments is the age of the donor cells (E13.5 and E12.5, respectively). Taking into account that most GP neurons are born between E10.5 and E12.5 (Fig. 2*C*) (Marchand and Lajoie, 1986), it is conceivable that this difference may account for the disparity in the results.

In addition to the MGE, our experiments revealed that neurons in the GP also derived from the POA and the LGE (Fig. 9). Fate-mapping analyses using *Dbx1–Cre* and *Pax6–Cre* mice provide direct evidence that some GP neurons are born outside the MGE, because neither one of these genes is expressed within this region (Flames et al., 2007). Analysis of *Dbx1–Cre* mice revealed that a small percentage of POA progenitors contribute to the complement of GP neurons. It is possible, however, that this analysis may underestimate the contribution of the POA to the GP, because *Dbx1* is only expressed by some progenitor cells within this region (Flames et al., 2007). It should be noted, however, that neurons expressing *Nkx5-1*, which identifies another neuronal lineage emanating from the POA (Gelman et al., 2009), do not populate the GP. Similarly, Cre expression is mosaic in our
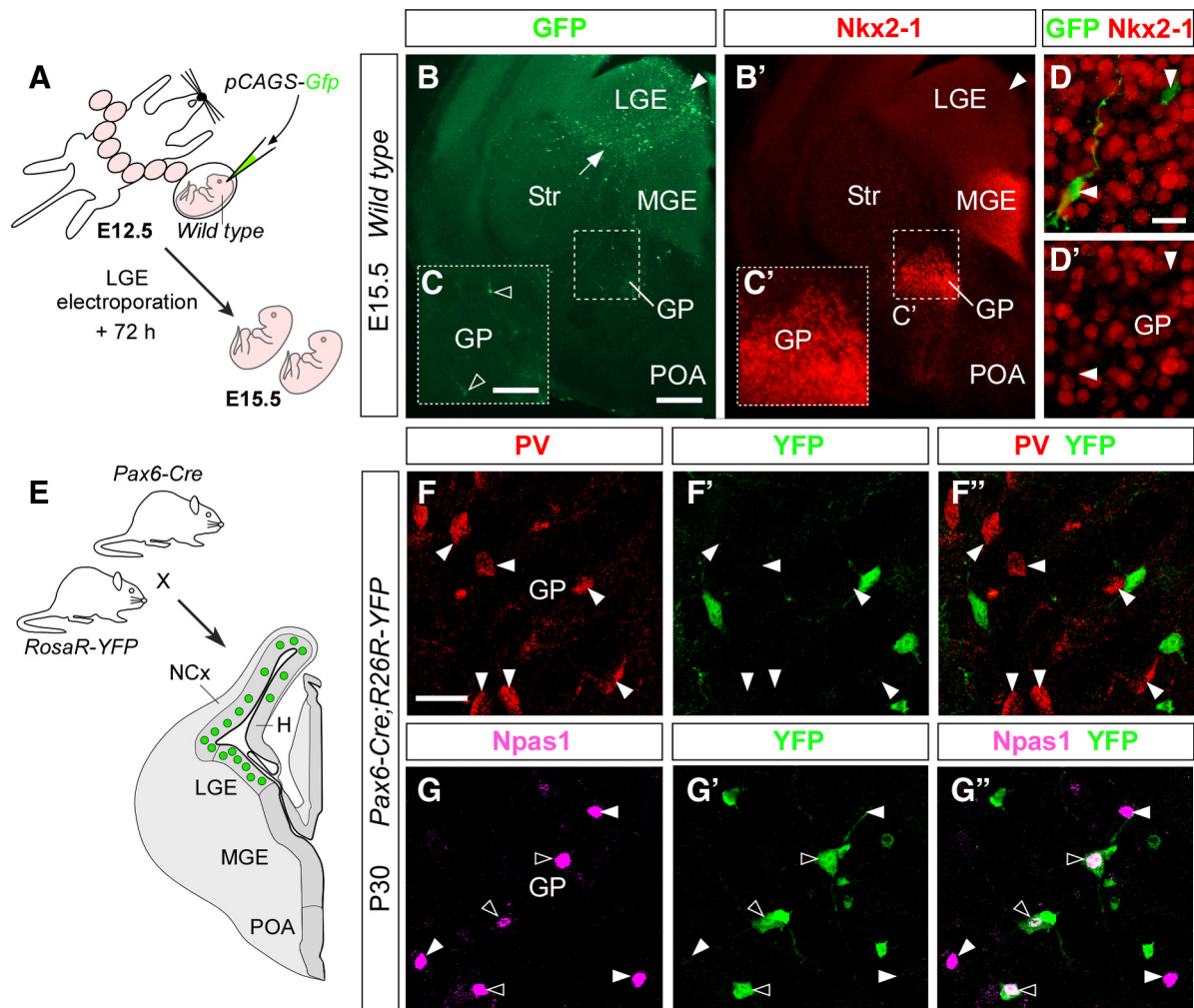
Figure 7. Origin of GP neurons in the LGE. ***A***, Schematic of the experimental design. ***B–C′***, A representative case of the distribution of GFP-expressing (***B***, ***C***) and Nkx2-1-expressing (***B′***, ***C′***) cells in a coronal section through the telencephalon of an E15.5 embryo in which the LGE was electroporated at E12.5. The white arrowhead in ***B*** and ***B′*** indicates the place of electroporation. Note that most cells derived from the LGE seem to migrate radially toward the developing striatum (arrow), but a few appear to turn ventrally toward the GP. ***C*** and ***C′*** are high-magnification images of the GP from the boxed area shown in ***B*** and ***B′***, respectively. ***D***, ***D′***, Images of representative cells found in the GP after electroporation in the LGE. ***E***, Schematic of the experimental design, indicating the progenitor domains labeled in *Pax6–Cre* mice. ***F–G″***, Coronal sections through the telencephalon of P30 *Pax6–Cre;Rosa26R–YFP* mice showing coexpression of PV (***F***, ***F″***) and Npas1 (***G***, ***G″***) with YFP (***F′***, ***F″***, ***G′***, ***G″***) in the GP. Open arrowheads point to examples of double-labeled cells, whereas filled arrowheads point to neurons that express only one of the markers. Scale bars: ***B***, ***B′***, 250 μm; ***C***, ***C′***, 100 μm; ***D***, ***D′***, 25 μm; ***F–G″***, 50 μm. Str, Striatum; NCx, neocortex.

Pax6–Cre mice, and therefore we could only trace a fraction of the derivatives of the LGE. Because progenitor cells throughout the pallium also express *Pax6*, it is conceivable that Npas1$^+$ neurons may also derive from this region. Indeed, a recent study has suggested that pallial progenitors derived from an *Emx1* lineage may give rise to inhibitory cells in the striatum and amygdala (Cocas et al., 2009). Nevertheless, pallial cells do not seem to generate many GABAergic cells (Chapouton et al., 1999; Stoykova et al., 2000; Toresson et al., 2000), and a recent report has shown that *Emx1–Cre* mice also induce recombination in the subpallium (Waclaw et al., 2009). Another possible origin for GP neurons derived from the *Pax6–Cre* lineage is the preoptic–hypothalamic area (POH), a territory adjacent to the POA (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). In summary, both the POA and the LGE seem to give rise to neurons that populate the adult GP, although other sources, such as the pallium and POH, may also contribute to the neuronal diversity found in this nucleus.

The analysis of *Nkx2-1* embryos provided additional support to the hypothesis that many GP neurons derived from the LGE.

Previous studies have shown that loss of *Nkx2-1* leads to a re-specification of MGE progenitor cells in a temporally regulated manner, which results in the ectopic production of striatal projection neurons and some classes of cortical interneurons (Sussel et al., 1999; Butt et al., 2008). Because these neurons are thought to originate from different rostrocaudal levels of the LGE, these studies demonstrated that the LGE expands at the expense of MGE progenitors in the absence of Nkx2-1 function. The finding that the population of Npas1-expressing GP neurons is preserved in *Nkx2-1* mutants therefore strongly suggests that many of these neurons derive from the LGE. The precise origin of Npas1-expressing GP neurons within the LGE remains to be determined, although previous *in utero* transplantation experiments suggest that these neurons may originate from the caudal aspect of the LGE, also known as the caudal ganglionic eminence (CGE) (Nery et al., 2002). This region of the subpallium also expresses *Pax6* and has been shown previously to be the origin of many tangentially migrating GABAergic neurons (Nery et al., 2002; Butt et al., 2008). Thus, it is possible that Npas1$^+$ neurons in the GP primarily originate from the CGE. Alternatively, these neu-

rons may derive from the entire rostrocaudal extent of the subpallial *Pax6* territory (i.e., LGE + CGE).

The discovery of multiple developmental origins for GP neurons suggests that neuronal diversity within this nucleus is relatively high (Fig. 9). Indeed, several classes of GABAergic projection neurons have been described based on various morphological and physiological criteria (Staines and Fibiger, 1984; Kita, 1994, 2007; Kita and Kitai, 1994; Bevan et al., 1998; Kita and Kita, 2001). For example, GP neurons project to other structures in the basal ganglia circuitry, such as the endopenduncular nucleus, the subthalamic nucleus, and the substantia nigra, but not all of them have the same specific synaptic target. In addition, approximately one-third of GP neurons also project to the striatum, in which they may contact either striatal projection neurons or interneurons (Bevan et al., 1998; Kita, 2007). GP neurons projecting to the striatum are typically immunonegative for PV, whereas nonstriatal projection GP neurons express PV (Kita and Kita, 2001). Thus, neuronal diversity of GP neurons, as revealed by their heterogeneous projection patterns and molecular features, seems to reflect the variety of progenitor pools involved in the generation of this structure.

Possible mechanisms controlling the migration of globus pallidus neurons
There are no reports on the possible guidance mechanisms used by GP neurons to assemble in the basal telencephalon. The structure of the radial glia in the developing telencephalon, as revealed by RC2 staining (Misson et al., 1988), strongly

suggests that only GP neurons derived from the MGE may use radial glia as their principal mode of migration. In contrast, LGE- and POA-derived GP neurons must necessarily reach their final position after migrating at least part of their trajectory following a tangential route.

The early neurogenesis of PV-expressing GP neurons (Fig. 2*C*) (Marchand and Lajoie, 1986) indicates that this population of cells may establish the foundation of this nucleus, with the remaining populations of GP neurons arriving afterward. In this model, cells derived from the MGE would first colonize the re-
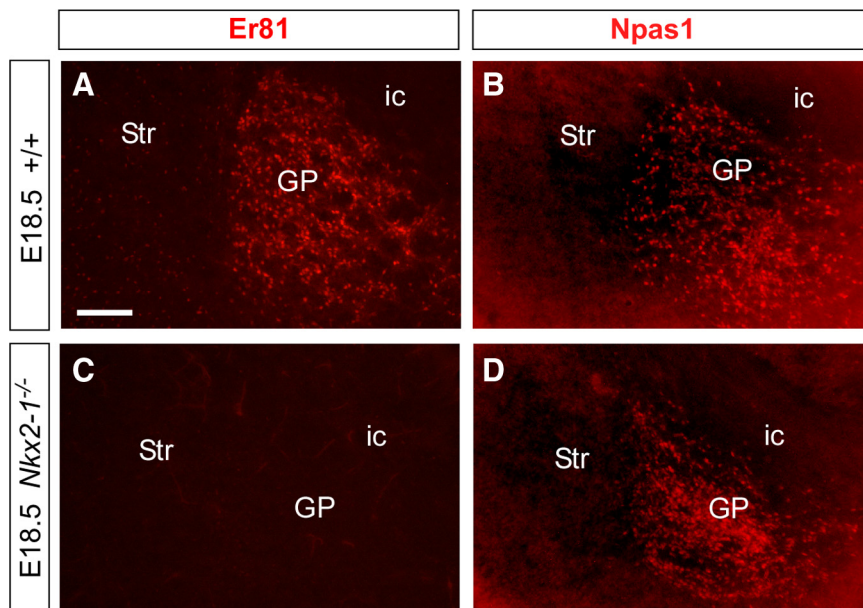

Figure 8. Npas1-expressing cells are present in the globus pallidus of *Nkx2-1* mutants. *A–D*, Coronal sections through the telencephalon of E18.5 wild-type (*A*, *B*) and *Nkx2-1* mutant (*C*, *D*) fetuses showing the distribution of Er81-expressing (*A*, *C*) and Npas1-expressing (*B*, *D*) cells in the GP. Scale bar, 250 μm. Str, Striatum; ic, internal capsule.

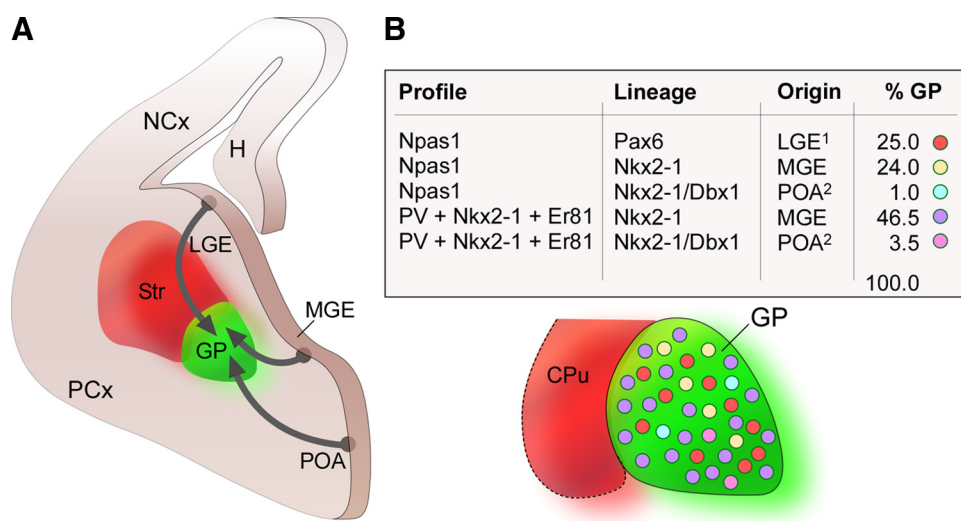| Profile	Lineage	Origin	% GP
Npas1	Pax6	LGE[1]	25.0
Npas1	Nkx2-1	MGE	24.0
Npas1	Nkx2-1/Dbx1	POA[2]	1.0
PV + Nkx2-1 + Er81	Nkx2-1	MGE	46.5
PV + Nkx2-1 + Er81	Nkx2-1/Dbx1	POA[2]	3.5
			100.0

Figure 9. Neuronal diversity in the globus pallidus emerges from different and distant progenitor pools. *A*, Schematic representation of a transversal hemisection through an E13.5 developing telencephalon, depicting the putative routes of migration of GP neurons from their origins to their final destination. *B*, Schematic representation of neuronal diversity in the GP, based on the molecular profile of its constituents and their differential origin. 1, The contribution of the *Pax6*-expressing territory (LGE proper and/or CGE) to the population of Npas1 + neurons is inferred from the fate-mapping analysis of *Nkx2-1–Cre;Rosa26R–YFP* mice, because expression of *Cre* in *Pax6 –Cre* mice is mosaic and does not label all LGE progenitors. In addition, it should be noted that the POH also expresses *Pax6* (supplemental Fig. S2, available at www.jneurosci.org as supplemental material), and therefore some Npas1 + neurons might derive from this region. 2, The contribution of the POA to the GP might be underestimated, because progenitors other than those expressing *Dbx1* may give rise to GP neurons. If this were the case, then the contribution of the MGE to the GP would be overestimated. H, Hippocampus; CPu, caudoputamen nucleus; PCx, piriform cortex; Str, striatum.

gion in which the GP would eventually form, and then neurons derived from the LGE and POA would join this structure. This would suggest that GP pioneer neurons could provide guidance cues for the tangentially migrating component of the GP, similar to what has been proposed for striatal neurons (Marín et al., 2000). Alternatively, each population of GP neurons might be directed to independently assemble in the caudomedial aspect of the developing striatum. This would be consistent with the finding that LGE-derived GP neurons aggregate in the correct location in the absence of MGE- and POA-derived GP neurons, as observed in *Nkx2-1* mutants. Thus, each population of neurons may rely on independent guidance mechanisms to assemble into the GP.

Nuclear assembly in the developing telencephalon

Our understanding of the formation of laminar structures in the telencephalon, such as the neocortex or the hippocampus, has massively expanded over the past 10 years (Gupta et al., 2002; Marín and Rubenstein, 2003; Ayala et al., 2007). In the cortex, projection neurons arise to their final destination through radial migration, which serves to maintain relatively stable topological relationships between the ventricular zone and the underlying mantle (Rakic, 1988, 2007). In contrast, interneurons reach the cortex through a long tangential migration (Corbin et al., 2001; Métin et al., 2006), which has led to the suggestion that this mechanism serves to increase neuronal diversity in the developing brain (Marín and Rubenstein, 2001). In the basal telencephalon, at least two nuclear structures seem to develop following the same principles. In the striatum, projection neurons appear to migrate radially from the LGE (Halliday and Cepko, 1992; Hamasaki et al., 2001), whereas interneurons reach their target from the MGE by tangential migration (Marín et al., 2000). Similarly, recent work has shown that the medial nucleus of the amygdala also forms by gathering radially migrating projection neurons from the LGE and tangentially migrating interneurons from the POA (Carney et al., 2006; Hirata et al., 2009).

Our study suggests that the assembly of forebrain nuclei might require a more complex set of events. Formation of the GP involves merging several different populations of cells that eventually will develop into distinct classes of projection neurons. This is relatively common to other forebrain nuclei, for which distinct classes of projection neurons have been described. In the striatum, for example, "matrix" and "striosome" projection neurons are generated at different times and have distinct connectivity patterns (van der Kooy and Fishell, 1987; Gerfen, 1992). However, what is remarkable about the GP is that different classes of projection neurons seem to derive from relatively distant progenitor pools, and many of them might reach their final destination through tangential migration. Thus, our results suggest that tangential migration in the forebrain is not limited to interneurons but also involves projection neurons. In addition, our experiments imply that the formation of topographical connections is not directly linked to the process of radial migration, because organized connectivity also emerges from brain structures with very elaborated patterns of nuclear assembly, such as the GP.

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