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Characterization of a new *Gsx2-Cre* line in the developing mouse telencephalon

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Summary

In this study, we generated a transgenic mouse line driving *Cre* and *EGFP* expression with two putative *cis*-regulatory modules (CRMs) (i.e. *hs687* and *hs678*) upstream of the homeobox gene *Gsx2* (formerly *Gsh2*), a critical gene for establishing lateral ganglionic eminence (LGE) identity. The combination of these two CRMs drives transgene expression within the endogenous *Gsx2* expression domains along the anterior-posterior neuraxis. By crossing this transgenic line with the *Rosa^{tdTomato}* (*Ai14*) reporter mouse line, we observed a unique recombination pattern in the lateral ventral telencephalon, namely the LGE and the dorsal half of the medial GE (MGE), but not in the septum. We found robust recombination in many cell types derived from these embryonic regions, including olfactory bulb (OB) and amygdala interneurons and striatal projection neurons from the LGE, as well as cortical interneurons from the MGE and caudal GE (CGE). In summary, this transgenic mouse line represents a new tool for genetic manipulation in the LGE/CGE and the dorsal half of MGE.

Keywords

ventral telencephalon; ganglionic eminence (GE); cre-mediated recombination; olfactory bulb (OB); striatum; amygdala

INTRODUCTION

The LGE and CGE represent major sources of many neuronal types in the basal telencephalon and cortex (Rubenstein and Campbell, 2013). Previous studies have demonstrated that many interneurons in the OB are generated in the embryonic dorsal LGE (dLGE) and its postnatal derivative dorsal lateral (dl) subventricular zone (SVZ) (Stenman et

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al., 2003; Waclaw et al., 2006; López-Juárez et al., 2013). On the other hand, striatal projection neurons are exclusively generated by progenitors in the ventral LGE (vLGE) (Stenman et al., 2003). The homeobox gene *Gsx2* is highly expressed in LGE progenitors and is critical for the establishment of dorsal-ventral patterning in the telencephalon (Szucsik et al., 1997; Rubenstein and Campbell, 2013) as well as the temporal specification of neuronal and glial subtypes at embryonic (Waclaw et al., 2009; Chapman et al., 2013) and adult stages (López-Juárez et al., 2013).

The molecular mechanisms that regulate *Gsx2* expression in the telencephalon are not well understood. Recently, Visel et al. (2009, 2013) described ChIP-seq data of p300, an acetyltransferase associated with active enhancers, from the E11.5 forebrain and annotated putative enhancers, a number of which have been used to generate transgenic *Cre* mouse lines used in forebrain fate mapping studies (Pattabiraman et al., 2014). Two of these enhancers were upstream of *Gsx2* and drove reporter activity patterns similar to that of *Gsx2* in the developing forebrain, hindbrain and spinal cord (VISTA Enhancer Browser; Visel et al., 2013). In this study, we generated a transgenic mouse line in which *Cre-IRES-EGFP* (*CIE*) was driven by these two putative human CRMs of *Gsx2* (*hs687* and *hs678*), termed *Gsx2e-CIE* and characterized the recombination patterns in *Gsx2*-expressing regions by crossing it with the *Rosa^{tdTomato}* (*Ai14*) reporter line (Madisen et al., 2010).

RESULTS AND DISCUSSION

hs687/hs678 drive transgene expression within endogenous *Gsx2*-expressing regions

Gsx2 is expressed in multiple regions in the CNS along the anterior-posterior (AP) axis, both embryonically and postnatally (Szucsik et al., 1997; Kriks et al., 2005; López-Juárez et al., 2013); however, the mechanisms that regulate its expression are not fully understood. Recent studies have identified two conserved potential enhancer elements upstream of *Gsx2*, *hs687* and *hs678*, that exhibit enhancer activity in the forebrain and hindbrain, respectively (VISTA Enhancer Browser; Visel et al., 2009; 2013) (Fig. 1A). In the mouse genome, the 687 element (http://enhancer.lbl.gov/cgi-bin/imagedb3.pl?form=presentation&show=1&experiment_id=687&organism_id=1) is located 62kb upstream of *Gsx2*, within the third intron of the *Chic2* gene. This element represents a strong candidate as a telencephalon CRM for *Gsx2* as it drives transgene expression within the LGE, however, on its own, it does so in a somewhat mosaic manner (Visel et al., 2013 and our unpublished data). The 678 element (http://enhancer.lbl.gov/cgi-bin/imagedb3.pl?form=presentation&show=1&experiment_id=678&organism_id=1), on the other hand, is 4kb upstream of *Gsx2* (Fig. 1A) and, on its own, drives transgene expression only in hindbrain regions. Given their genomic organization, we reasoned that combining the two elements in the same order as they appear upstream of *Gsx2* may lead to a more faithful recapitulation of endogenous *Gsx2* gene expression. To do this, we constructed a β -globin minimal promoter-driven *Cre-IRES-EGFP* (*CIE*) under the control of both *hs687* and *hs678* (Fig. 1B), hereafter called, *Gsx2e-CIE*.

Gsx2e-CIE embryos showed robust EGFP signal in *Gsx2*-expressing regions along the AP axis including LGE, diencephalon, hindbrain and spinal cord (Fig. 1C). We found robust EGFP in the LGE at E10.5, as determined by *Gsx2* protein co-staining (Fig. 1D, E), with the

earliest LGE transgene expression starting at E10. Additionally, we observed scattered EGFP in the dorsal half of the *Nkx2.1*⁺ MGE (Fig. 1D–F). Overall, the EGFP expression pattern was consistent with endogenous *Gsx2* dorsal-high-ventral-low expression pattern (Yun et al., 2001; Waclaw et al., 2009). By E12.5, a mosaic dorsal-high-ventral-low gradient of EGFP was present within the *Gsx2*-expressing domain. We found the strongest EGFP in the dLGE cells displaying radial glial morphology, and many EGFP⁺ cells in the VZ co-expressed *Gsx2*, however, only scattered EGFP⁺ cells were detected in the vLGE and MGE (Fig. 1I, J). At later stages (e.g. E15 onward), EGFP⁺ cells became scarce in the *Gsx2*⁺ VZ (data not shown), suggesting that other CRMs may play a larger role in regulating *Gsx2* expression late in development. *Gsx2* is also expressed in the developing diencephalon, hindbrain and spinal cord (Szucsik et al., 1997; Kriks et al., 2005). Appropriately, we observed EGFP⁺ cells in the diencephalon, hindbrain and spinal cord of *Gsx2e-CIE* embryos (Fig. 1G, H and data not shown).

***Gsx2e-CIE;Ai14* fate map**

To investigate the recombination pattern driven by the *Cre* transgene, we crossed the transgenic line to a *Rosa^{tdTomato}* (*Ai14*) reporter line (Madisen et al., 2010). From whole-mount view, we observed 3 out of 19 *Gsx2e-CIE;Ai14* embryos (E12.5 or E15.5) collected displaying widespread tdTomato expression and these were not analyzed further; whereas the other 16 showed a recombination pattern that was consistent with *Gsx2* expression along the neuraxis. Indeed, we observed a uniform tdTomato expression throughout the LGE VZ as well as the adjacent mantle zone at E12.5 (Fig. 2A–D). We also found strong tdTomato signal in the E12.5 CGE (Fig. 2E, F). Interestingly, the recombination pattern driven by *Gsx2e-CIE* was somewhat distinct from that of the previously described *Gsh2-iCre* line (Kessaris et al., 2006), which, in our hands, resulted in a more mosaic recombination within the LGE VZ when crossed with the *Ai14* mice (Supplementary Fig. 1). These latter mice were made using a PAC that includes the proximal 678 CRM and extends 3' of the *Gsx2* locus, but lacks the distal 687 enhancer (Kessaris et al., 2006).

At E15.5, rather uniform recombination remained in the VZ of the LGE (Fig. 2G, I) and CGE (Fig. 2K). Again, many *Gsx2*⁺ cells were also tdTomato⁺ (Fig. 2H, J, L). We also observed strong tdTomato signal in the SVZ and the forming striatum. The CGE is thought to be a major source of cortical interneurons (Miyoshi et al., 2010). Not surprisingly, we found tdTomato⁺ cells migrating from the CGE into the developing cortex (see below). A similar recombination pattern was observed in the P0 LGE and CGE (Fig. 2M–R). Interestingly, despite the expression of *Gsx2* in the septal VZ (Fig. 2H, N), the *Gsx2*⁺ septal VZ was devoid of recombination (Fig. 2G–R).

We next examined the recombination pattern in the MGE of *Gsx2e-CIE;Ai14* embryos. *Nkx2.1* is expressed by MGE cells and determines MGE identity (Sussel et al., 1999). In the E12.5 embryos, recombination was enriched in the dorsal half of the MGE but not in the ventralmost portion (Fig. 3A–F). Accordingly, the preoptic area (POA) was devoid of recombination (Fig. 3D–F). This recombination pattern was maintained in the E15.5 MGE (Fig. 3G–I); however, relatively uniform recombination was detected in the caudal MGE (Fig. 3J–L).

The MGE serves as an important origin of cortical interneurons (Wonders and Anderson, 2006). In the cortex, MGE-derived interneurons express *Lhx6* (Lavdas et al., 1999) and we observed many tdTomato⁺ cells expressing *Lhx6* in the P0 cortex (Fig. 3P–R). Most tdTomato⁺ cells in the superficial layers did not co-express *Lhx6*, presumably corresponding to interneurons derived from CGE (Miyoshi et al., 2010) (Fig. 3P–R).

The LGE and dLSVZ are important sources of OB interneurons embryonically and postnatally, respectively (Stenman et al., 2003; Waclaw et al., 2006; López-Juárez et al., 2013). Many tdTomato⁺ OB interneurons could already be found in the P0 OB (data not shown). Moreover, recombined cells were also observed in the P21 dLSVZ, along the rostral migratory stream (RMS) (Supplemental Fig. 2A), and within the glomerular layer (GL) and granule cell layer (GCL) of the OB (Fig. 4A–D). To confirm the interneuron identity of tdTomato⁺ OB cells, we immunostained the fate-mapped OB with an antibody against Sp8, a transcription factor expressed by many OB interneurons (Waclaw et al., 2006) and found that many tdTomato⁺ cells in the GL and GCL co-expressed Sp8 (Fig. 4B and C). We also found many tdTomato⁺ cells in the P21 dLSVZ expressed Sp8, corresponding to newborn neuroblasts (Fig. 4D inset). Besides OB interneurons, the dLGE produces GABAergic intercalated cells (ITCs) cells of the amygdala (Waclaw et al., 2010). Indeed most Foxp2⁺ ITCs were also tdTomato⁺ (Fig. 4G). Additionally, recombined cells were found in the vLGE-derived central amygdala (Waclaw et al., 2010) (Fig. 4G).

We also found strong tdTomato expression in the vLGE-derived striatal cells (Fig. 2M–R and Fig. 4D). At P21, many tdTomato⁺ cells in striatum expressed striatal projection neuron markers DARPP-32 and Foxp1 (Fig. 4E, F). In fact, about 75% of the Foxp1⁺ striatal neurons were tdTomato⁺. Additionally, striatal projection neurons forming both the direct and indirect output pathways were robustly recombined (Supplemental Fig. 2B).

GABAergic cortical interneurons are derived from subpallial regions and tangentially migrate to the cortex, comprising about 25% of the total cortical neuronal population (Marin and Rubenstein, 2001; Wonders and Anderson, 2006). Interestingly, only a portion of the *Lhx6*⁺ cortical interneuron precursors migrating from the MGE to the cortex were tdTomato⁺ (Fig. 3P–R and data not shown), suggesting that only a subpopulation(s) of the cortical interneurons were recombined. Two major non-overlapping cortical interneuron subtypes are produced in the MGE, namely the PV⁺ and SST⁺ interneurons (Xu et al., 2004; Fogarty et al., 2007). About 36% of the PV⁺ cortical interneurons were recombined, corresponding to about 20% of the tdTomato⁺ cells in the cortex (Fig. 5A), while 56% of the SST⁺ interneurons were recombined (Fig. 5B). Previous studies demonstrated that the CGE gives rise to about 30% of the cortical interneuron population of distinct subtypes from those derived from the MGE, notably by the lack of SST expression (Nery et al., 2002; Xu et al., 2004; Miyoshi et al., 2010; Gelman and Marín, 2010). We noticed robust recombination in the CGE (Fig. 2), and after double staining the P21 cortex for SST and CR, we found 66% of the CR⁺;SST[−] (i.e. CGE-derived) interneurons co-expressed tdTomato (Fig. 5D). Furthermore, many Reelin⁺;SST[−] interneurons were also tdTomato⁺ (Fig. 5E). VIP⁺ cortical interneurons are exclusively produced by CGE (Miyoshi et al., 2010) and we found about 75% of the VIP⁺ interneurons were tdTomato⁺ (Fig. 5C). Similarly, MGE- and CGE-derived hippocampal interneurons were recombined (data not shown). Taken together, our results

indicated efficient but selective recombination of both MGE- and CGE-derived interneurons in the *Gsx2e-CIE;Ai14* mice.

The MGE also gives rise to interneurons in the striatum and projection neurons in the GP (Sussel et al., 1999; Marín et al., 2000; Xu et al., 2008; Flandin et al., 2010). No Nkx2.1⁺ cells in the P0 striatum or GP were recombined (Fig. 3M–O). Accordingly, we found no ChAT⁺ (i.e. cholinergic) or NPY⁺ (GABAergic) interneurons co-expressing tdTomato at P21 (Fig. 6A, B). Likewise, no PV⁺ (GABAergic) interneurons in the striatum (Fig. 6C, D) or projection neurons in the GP were fate-mapped (Fig. 6C, E), supporting the notion that they derive from the ventral portion of the MGE (Flandin et al., 2010). In contrast, many ventral MGE-derived Nkx2.1⁺ cells were recombined in the forming GP of *Gsh2-iCre;Ai14* embryos (Supplementary Fig. 1E).

In summary, the *Gsx2e-CIE* mice provide a useful tool to recombine progenitors and their progenies from the LGE/CGE and dorsal portion of the MGE but not from the septum, therefore allowing regionally selective genetic manipulation in the ventral telencephalon. Moreover, the *Gsx2e-CIE* mice appear to recombine more robustly within the VZ of these ventral telencephalic regions than the existing *Gsh2-iCre* mice (Kessaris et al., 2006), thereby enabling more efficient genetic manipulation in early VZ progenitors.

METHODS

Animals

The *Gsx2e-Cre-IRES-EGFP (CIE)* transgenic lines were generated through pro-nuclear injection by CCHMC Transgenic Animal and Genome Editing Core Facility. The *687-678-CIE* plasmid was constructed using the human *687* (*hs687*, ~1.2kb) and *678* (*hs678*, ~0.8kb) sequences (Visel et al., 2009; 2013). The *hs687* and *hs678* plasmid constructs were kindly provided by Axel Visel (Lawrence Berkley National Laboratory). The *687-CIE* was constructed by inserting *hs687* into a gateway-cloning vector containing *CIE*. The *687-678-CIE* construct was then generated by inserting the *hs678* at the 3' end of *hs687* (Fig. 1A) by infusion cloning (Clontech). The *687-678-CIE* fragment was released by XhoI and NaeI, gel purified, and proceeded for pro-nuclear injection. The *Gsx2e-CIE* mice were maintained in outbred background and genotyped by the following primers: Cre: JaxCre-5' (5'-GCGGTCTGGCAGTAAAACTATC-3') and JaxCre-3' (5'-CCATGAGTGAACGAACCTGG-3'); EGFP: JaxGFP-5' (5'-AAGTTCATCTGCACCACCG) and JaxGFP-3' (5'-TCCTTGAAGAAGATGGTGCG). The *Rosa-tdTomato (Ai14)* line was purchased from the Jackson Laboratory (stock #007908) and genotyped by the following primers: Rosa-tdTomato-5' (5'-GGCATTAAAGCAGCGTATCC-3') and Rosa-tdTomato-3' (5'-CTGTTCCTGTACGGCATGG-3') (Madisen et al., 2010). *Gsh2-iCre* mouse was obtained from Jackson Laboratory (stock #025806) and genotyped by the following primers: 21218 (5'-CAGGTTTTGGTGCACAGTCA-3') and 23315 (5'-ACGTGAGGACCAACCCTCT-3') (Kessaris et al., 2006).

Four transgenic *Gsx2e-CIE* founders were generated through pronuclear injection and transgene expression was analyzed by visualization of EGFP. One founder male failed to

breed, preventing further analysis of the line, and the offspring of the two other founders showed no or only scattered EGFP in the *Gsx2*-expressing domains. The fourth founder generated a line, which showed robust EGFP signal in the *Gsx2*-expressing regions along the AP axis and this line represents the focus of the current report and will be available to the research community.

For all crosses, the morning of the vaginal plug observed was designated E0.5. E10.5 and E12.5 embryos were fixed in 4% paraformaldehyde (PFA) for 3 hours and 6 hours, respectively. E15.5 and P0 brains were fixed in 4% PFA overnight and were extensively rinsed in PBS. Brains were cryoprotected in 30% (E15.5 or younger) or 20% (P0) sucrose before being sectioned on a cryostat at 12µm.

Postnatal brains were collected at P21. Brains were dissected out from the skull and fixed in 4% PFA overnight. Brains were extensively rinsed in PBS and cryoprotected in 20% sucrose. 20µm coronal sections were then collected on a cryostat. Sagittal sections were obtained on a sliding microtome at 35µm.

Immunohistochemistry (IHC)

The sections were blocked in 10% normal donkey serum (NDS) before carrying out IHC as described in Waclaw et al., 2006. The following antibodies were used: goat anti-calretinin (CR) (1:2000, Millipore); rabbit anti-ChAT (1:500, Millipore); goat anti-DARPP-32 (1:500, Santa Cruz Biotechnology); rabbit anti-dsRed (1:500, Clontech); rabbit anti-Foxp1 (1:4000, abcam); rabbit anti-Foxp2 (1:5000, abcam); chicken anti-GFP (1:1000, Aves Labs); rabbit anti-Gsx2 (1:5000, Toresson et al., 2000); rabbit anti-Lhx6 (1:1000; a gift from Dr. Stewart A. Anderson, Children's Hospital of Philadelphia, Petros et al., 2015); rabbit anti-Nkx2.1 (1:500, Seven Hills Bioreagent); rabbit anti-NPY (1:500, Immunostar); rabbit anti-Parvalbumin (PV) (1:500, Swant); mouse anti-Reelin (1:500, Calbiochem); goat anti-Sp8 (1:8000, Santa Cruz Biotechnology); rabbit anti-somatostatin (SST) (1:500, millipore); rabbit anti-vasoactive intestinal polypeptide (VIP) (1:500, Immunostar). Secondary antibodies for fluorescent staining were: donkey anti-rabbit conjugated with alexa 488, alexa 647 or Cy3 (1:200, Jackson ImmunoResearch); alexa 488 conjugated donkey anti-chicken (1:200, Jackson ImmunoResearch); alexa 488 conjugated donkey anti-mouse (1:200, Jackson ImmunoResearch); donkey anti-goat conjugated with Cy3 or alexa 647 (1:200, Jackson ImmunoResearch). Fluorescent slides were covered with Fluoromount-G (SouthernBiotech). Confocal images were taken on NikonA1RGaAsP invert microscope or Nikon C2 confocal microscope.

Data analysis

Out of 30 *Gsx2e-CIE;Ai14* animals analyzed, we observed ectopic tdTomato⁺ cortical cells (few to moderate) in 12 animals. This ratio was similar to what we observed in *Gsh2-iCre;Ai14* embryos (1 out of 3) that showed ectopic tdTomato⁺ cortical cells. Interestingly, transgene expression (as determined by EGFP) in the *Gsx2e-CIE;Ai14* animals was consistently normal. Such variable recombination has been reported previously in a number of *Cre* lines and may be influenced by genetic background (Heffner et al., 2012).

Three P21 *Gsx2e-CIE;Ai14* animals were used for quantifying recombination among different neuronal populations. Both paracapsular and IA (intercalated mass of the amygdala) intercalated cells were counted for quantifying recombination in the amygdala. Cortical interneurons in the somatosensory cortex were quantified to represent recombination among interneuron subtypes. Images for quantification were captured with an Olympus BX50 epifluorescence microscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) 687 (red bar) and 678 (blue bar) are located in vicinity of *Gsx2* in the mouse genome and are conserved across vertebrates (from the UCSC genome browser). (B) Diagram of the strategy of the transgene construct. *Cre-IRES-EGFP* was driven by β -globin minimal promoter regulated by *hs687* and *hs678*. (C) EGFP from the transgene was present in the E10.5 telencephalon, diencephalon, hindbrain and spinal cord. (D and E) EGFP was highly expressed by *Gsx2*⁺ progenitor cells in the E10.5 LGE. (F) EGFP was weakly present in the dorsal portion of the *Nkx2.1*⁺ MGE at E10.5, MGE/LGE boundary was determined by *Nkx2.1* staining and marked by arrows. (G and H) EGFP from the transgene was detected in the E10.5 diencephalon and spinal cord. (I and J) EGFP was highly expressed by cells in the E12.5 dLGE but very mosaic EGFP was detected in the vLGE and MGE. Telencephalon, tel; diencephalon, di; hindbrain, hb; spinal cord, sc. Scale bar: 100 μ m.

Figure 2.

Gsx2e-CIE;Ai14 fate map in the developing LGE. (A–F) tdTomato was relatively uniformly distributed in the E12.5 LGE and CGE as well as rostrrodorsal MGE (see Fig. 3). (G and H) tdTomato was expressed by many VZ *Gsx2*⁺ progenitor cells and their SVZ derivatives in the E15.5 rostral dLGE but was absent in the septal VZ. (I–L) tdTomato was expressed throughout the E15.5 LGE and CGE. (M–R) tdTomato was uniformly expressed by P0 LGE *Gsx2*⁺ progenitors (insets in N, P and R) and striatal cells, however no tdTomato was present in the P0 septal VZ. Scale bar: 100 μ m.

Figure 3.

Gsx2e-CIE;Ai14 fate map in the developing MGE and its derivatives. (A–F) At E12.5, tdTomato was highly expressed by cells in the dorsal half of the MGE but was absent in the ventral half or POA. (G–L) tdTomato marked the dorsal portion of MGE (delimited by dashed line in H and I) at rostral level but the whole Nkx2.1⁺ domain in the caudal area at E15.5. (M–O) tdTomato was not expressed by MGE-derived Nkx2.1⁺ striatal interneuron and globus pallidus (GP) projection neurons in the P0 telencephalon. (P–R) A subpopulation of Lhx6⁺ cortical interneuron expressed tdTomato, corresponding to a portion of the tdTomato⁺ cells in the P0 cortex. GP, globus pallidus. Scale bar: 100 μm.

Figure 4.

Gsx2e-CIE;Ai14 fate map in LGE-derived neuronal populations. (A–C) tdTomato marked many Sp8⁺ interneurons in the P21 olfactory bulb glomerular layer, the granule cell layer and the rostral migratory stream. (D) tdTomato was highly expressed by cells in the dlSVZ (inset), striatum, olfactory tubercle, nucleus accumbens and septal parenchyma in the P21 brain. (E and F) tdTomato⁺ cells in the P21 striatum expressed projection neuron markers DARPP-32 and Foxp1. (G) tdTomato⁺ cells were enriched in the CA and IA of the P21 amygdala. Paracapsular and IA tdTomato⁺ cells were intercalated cells (ITCs) as determined by Foxp2 staining. Glomerular layer, GL; granule cell layer, GCL; rostral migratory stream, RMS; external plexiform layer, EPL; mitral cell layer, ML; olfactory tubercle, OT; nucleus accumbens, acb; lateral amygdala, LA; basolateral amygdala, BLA; central amygdala, CA; intercalated mass of the amygdala, IA. Scale bar: 100 μm (A–C, inset in D, E–G), 500 μm (D)

Figure 5.

Gsx2e-CIE;Ai14 fate map among cortical interneurons. (A and B) Many PV- and SST-expressing cortical interneurons in the P21 somatosensory cortex were fate-mapped by tdTomato. (C) CGE-derived VIP⁺ interneuron enriched in the superficial cortical layers were heavily fate-mapped by tdTomato. (D) Many CR⁺;SST⁻ CGE-derived cortical interneurons were also tdTomato⁺. (E) tdTomato was detected in many Reelin⁺SST⁻ cortical interneurons (arrowheads). Some Reelin⁺;SST⁺ interneurons were also marked by tdTomato (arrow). Scale bar: 100 μ m.

Figure 6.
Gsx2e-CIE;Ai14 fate map in subcortical areas. (A and B) tdTomato failed to mark cholinergic ChAT⁺ interneurons and GABAergic NPY⁺ interneurons in the P21 striatum. (C–E) PV⁺ striatal interneurons and GP projection neurons were not fate-mapped by tdTomato. Scale bar: 100 μm.