NeuroGT: A brain atlas of neurogenic tagging CreER drivers for birthdate-based classification and manipulation of mouse neurons

Graphical abstract

Highlights

- We generate four CreER mouse drivers to tag neurons on the basis of time of neurogenesis
- The drivers enable birthdate-based classification and manipulation of neurons
- We develop NeuroGT, an image atlas of tagged neurons across the entire brain
- Through NeuroGT, researchers can find driver lines suitable for their own research

Authors

Tatsumi Hirata, Yukako Tohsato, Hiroya Itoga, ..., Sanae Oka, Toshihiko Fujimori, Shuichi Onami

Correspondence
tathirat@nig.ac.jp

In brief

Hirata et al. develop neurogenic tagging CreER driver mice and the NeuroGT database to showcase the mouse lines. The resource can be used to classify neurons on the basis of their generation timing and manipulate the classified neuron subsets for research purposes.

https://ssbd.riken.jp/neurogt/
NeuroGT: A brain atlas of neurogenic tagging CreER drivers for birthdate-based classification and manipulation of mouse neurons

Tatsumi Hirata,1,2,8 Yukako Tohsato,3,4 Hiroya Itoh,4 Go Shioi,5 Hiroshi Kiyonari,5 Sanae Oka,6 Toshihiko Fujimori,2,6 and Shuichi Onami4,7

1Brain Function Laboratory, National Institute of Genetics, 1111 Yata, Mishima 411-8540, Japan
2The Graduate University for Advanced Studies, SOKENDAI, Hayama, Japan
3Computational Biology Laboratory, Faculty of Information Science and Engineering, Ritsumeikan University, 1-1-1 Noji-higashi, Kusatsu, Shiga 525-8577, Japan
4Laboratory for Developmental Dynamics, RIKEN Center for Biosystems Dynamics Research, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan
5Laboratory for Animal Resources and Genetic Engineering, RIKEN Center for Biosystems Dynamics Research, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan
6Division of Embryology, National Institute for Basic Biology, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan
7Life Science Data Sharing Unit, RIKEN Information R&D and Strategy Headquarters, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan
8Lead contact

*Correspondence: tathirat@nig.ac.jp

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**SUMMARY**

Neuronal birthdate is one of the major determinants of neuronal phenotypes. However, most birthdating methods are retrospective in nature, allowing very little experimental access to the classified neuronal sub-sets. Here, we introduce four neurogenic tagging mouse lines, which can assign CreER-loxP recombination to neuron subsets that share the same differentiation timing in living animals and enable various experimental manipulations of the classified subsets. We constructed a brain atlas of the neurogenic tagging mouse lines (NeuroGT), which includes holistic image data of the loxP-recombined neurons across the entire brain.

**INTRODUCTION**

During development, neurons are generated over a protracted time window. Mounting evidence shows that neurogenic timing, which is often referred to as the neuronal birthdate, has immense impacts on neuronal phenotypes. In the neocortex, the birthdate determines layer positioning, connection patterns, and molecular and physiological properties of neurons (McConnell, 1989; Lodato and Arlotta, 2015). The chronological specification of neuronal fates is not a unique property of the neocortex but rather a generally conserved strategy in various nervous systems for generating neuronal diversity (Suzuki and Hirata, 2013). Even without affecting the intrinsic molecular differences, the birth timing itself could cause differential neuronal phenotypes because neurons with different birthdates are influenced by a changing environment (Hirata and Iwai, 2019). Because of its intimate relationships with various neuronal phenotypes, neuronal birthdating has been a reliable standard for the classification of...
neurons in various nervous systems for over half a century (Bayer and Altman, 1987; Govindan et al., 2018).

Traditionally, neuronal birthdating has been performed by using nucleotide analogs, such as radioactive thymidine and, more recently, bromodeoxyuridine (BrdU) or 5-ethylnyl uridine (EdU), which are incorporated into the DNA during the S phase to mark the final round of the cell cycle. Although these techniques have substantially improved, they are still descriptive histological techniques to determine neuronal birthdates in sacrificed animals. Recently, we developed a neurogenic tagging method by which tamoxifen (TM)-dependent CreER (Feil et al., 1997) recombination was induced in olfactory bulb neurons in a birthdate-dependent manner (Hirata et al., 2019). Given that this technique is based on irreversible loxP recombination, the tag can be used later to explore the birthdate-classified neuronal subset in various ways.

This neurogenic tagging method uses driver mouse lines in which CreER is transiently expressed within a short neurogenic time window in various neurons. TM injection on a single day during E9.5–E18.5 induces loxP recombination only in cells expressing CreER. The mouse embryos were raised up to P7, and the brains were sampled for visualization of tagged neurons. The BAC genomic constructs recombined with the CreER cassette that were used to generate the four neurogenic tagging driver lines. Boxes show the exons, in which the shaded part represents originally protein-coding sequences and the white part untranslated sequences. The approximate lengths of the genome upstream and downstream of the CreER cassette are indicated under each construct. The bottom panel shows a schematic of the gene structure of the Tau-mGFP-nLacZ reporter. Both reporter proteins, mGFP and nls-bGal, were used to visualize tagged neurons.

To cover other brain regions, we developed three neurogenic tagging driver lines by using the neuronal differentiation genes neurog1, neurod1, and neurod4 (Figures 1B and 1C), all of which are basic-helix-loop-helix transcription factors that are transiently expressed during the maturation phase of neurons (Kim et al., 2011; Sudarov et al., 2011; Aprea et al., 2014). Together with the previous line developed by using neurog2, the collection of driver lines covers most of the mouse nervous systems. Our aim is to contribute this resource to the scientific community. To encourage the use of this resource, we have developed a brain atlas of neurogenic tagging mouse lines (NeuroGT). This database contains section images of the entire brain visualized for tagged neurons as well as their processes; these images were obtained from postnatal mice that received a single TM injection on each day during the neurogenic period. Researchers interested in particular brain regions can find appropriate mouse lines and tagging stages for their research purposes.

RESULTS

NeuroGT and neurogenic tagging driver mice

The neurogenic tagging drivers Neurog2CreER(G2A), Neurog1CreER(G1C), Neurod1CreER(D1B), and Neurod4CreER(D4A) were developed as described in STAR Methods and are deposited at the RIKEN Center for Biosystems Dynamics Research (RIKEN BDR) (Figures 1B and 1C). To showcase the tagged neuron images comprehensively, each neurogenic tagging line was crossed with Tau-mGFP-nLacZ mice (Hippemeyer et al., 2005), which is a global neuronal Cre reporter that expresses dual nucleus- and membrane-localized reporters under a constitutive neuronal promoter after the excision of the loxP-STOP-
loxP cassette (Figure 1B). Staged pregnant mice were then intraperitoneally injected with TM only once during the gestation stages at embryonic day 9.5 (E9.5)–E18.5 (hereafter called TM9.5–TM18.5, referring to TM injection stages), and the tagged offspring were delivered and raised up to postnatal day 7 (P7).

Figure 2 shows X-gal-stained images of whole-mount brains tagged by the four drivers at different TM stages. The tagged neurons stained blue by the nucleus-localized (nls)-βGAL reporter were distributed in different brain regions depending on the TM injection stages and on the driver lines. Some brain regions were tagged by multiple drivers, whereas others were tagged specifically only by a single driver (Figure 2 and Table 1). From the overall external appearance, a neurogenic wave was observed to migrate from posterior regions, such as the hindbrain, to the more anterior regions according to the TM injection stages (Figure 2). In the brain regions tagged by multiple drivers, the spatiotemporal patterns of tagging were basically similar (Table 1), indicating that all the driver lines capture a similar neurogenic time window in neurons.

To expose the internal structures, we collected and coronally sectioned brains tagged at different TM stages by individual drivers. The interspaced serial sections were antibody stained with diaminobenzidine (DAB) for either the nls-βGAL or the membrane-localized mGFP reporter. All these sections were converted into high-resolution digital images (e.g., Figures 6B–6D) and subgrouped into datasets according to three categories, namely the driver line, TM stage, and stained reporter (nls-βGAL or mGFP). Each dataset defined by the combination of the three categories contained 142–180 interspaced section images, which were aligned along the entire anterocaudal axis of the brain (approximately 1.2 mm in length). This coverage is sufficiently high to identify even a small nucleus or structure in the brain (Table 1).

All datasets of the high-resolution section images (835 GB of data for 13,538 images in 84 datasets including TM-negative controls) and whole-mount images shown in Figure 2 are available in the NeuroGT database (https://ssbd.riken.jp/neurogt/). Users can search for these datasets from a web browser by using terms that match the meta-information stored in the database, such as their identifier, driver name, TM stage, or reporter used for staining (Figure 3A). The search results are displayed as a list of links to the dataset page (Figure 3B). To enable this search and visualization, development of the NeuroGT was based on the SSSBD (Systems Science of Biological Dynamics) database (Tohsato et al., 2016). The dataset pages at the next stage allow users to download the high-resolution section images (Figure 3C) and interactively view their thumbnails together with the meta-information. The thumbnail images of nls-βGAL- and mGFP-stained sections are stacked separately in order along the anteroposterior axis (Figure 3D) and can be viewed sequentially by dragging the slider (Video S1). The search function for anatomical regions will be implemented in the future. Through NeuroGT, researchers can identify the drivers useful for their research and obtain them from RIKEN BDR (http://www2.cist.riken.jp/arg/TG%20mutant%20mice%20list.html).

Table 1 summarizes the brain regions that were tagged by individual drivers. When the tagged stages were compared with the reported birthdates determined in mice by using nucleotide analogs, the ranges were fairly consistent (Table 1). The exceptions might be the pontine nucleus and dorsal root ganglion. Considering the short time lag often observed between the final S phase and the expression of CreER (Florio et al., 2012; Toma et al., 2014; Hirata et al., 2019; see also this paper), the TM stages for labeling these regions slightly preceded the birthdates determined with nucleotide analogs, suggesting that the loxP recombination might have occurred in pre-mitotic neuronal progenitors in these regions. Table 1 lists only limited brain regions from areas containing extensive tagged neurons. The NeuroGT database can be used to access and visualize a specific brain region of interest.

The following sections describe the features of each driver line, focusing on specific regions that can be effectively tagged by a particular line.

**Neurog2CreER(G2A) driver using the neurog2 enhancer**

The Neurog2CreER(G2A) line tags the most extensive brain regions. Specifically, at TM10.5–TM12.5, sagittal stripes in the cerebellum were labeled (magenta arrowheads 1 in Figure 2A), reflecting birthdate-dependent generation of cerebellar Purkinje neurons (Hashimoto and Mikoshiba, 2003; Namba et al., 2011). TM injection between TM11.5 and TM17.5 heavily labeled olfactory bulb neurons, as reported previously (Hirata et al., 2019), and neurons in the central olfactory areas and the amygdala (blue arrowheads 2 in Figure 2A). The dense labeling of the piriform cortex at TM11.5–TM12.5 demarcated a sharp border from the neocortex that was labeled by later TM injections, as will be described in the next paragraph (see also Figure 4C). In the inferior colliculus of the midbrain, a neurogenic gradient from the lateral to the medio-caudal was marked at TM12.5–TM16.5 (brown arrowheads 3 in Figure 2A) as previously reported (Altman and Bayer, 1981). Labeling of the inferior colliculus was also observed in the same spatiotemporal pattern by other neurogenic tagging drivers (brown arrowheads 3 in Figures 2C and 2D; Table 1).

In cross-sections of the neocortex, the inside-out birth-order arrangement of neurons was clearly visible with the nls-βGAL reporter (Figure 4A). As expected from the neurog2 expression, only excitatory projection neurons, but not GABAergic inhibitory neurons, were tagged in the neocortex (Figures S1A–S1C). The mGFP reporter prominently labeled neuronal fibers, including axons and dendrites (Figures 4B–4F). In a close-up of the corpus callosum (Figure 4B), long axons projecting from the tagged neocortical neurons were visualized. Interestingly, the axons of the early-born (TM12.5–TM13.5) and late-born (TM16.5–TM17.5) cortical neurons formed dorsoventrally segregated fascicles within the corpus callosum, presenting a feature that would have been difficult to recognize by nucleotide-based birthdating. Previous studies have reported the dorsoventral segregation of callosal axons from the medial and lateral cortical areas (Piper et al., 2009; Nishikimi et al., 2011; Zhou et al., 2013). The present axon compartments formed by the early-born and late-born callosal neurons appeared to be different from those based on the distinct cortical areas (Figures S1D–S1G), adding complexity to the organization of the corpus callosum.

Another unique feature of the neocortex was the patchy areal distribution of superficial neurons in the layer II/III tagged around
Figure 2. Neurogenic-tagged neurons in whole-mount brains

Ventral and dorsal views of P7 brains tagged by Neurog2\textsuperscript{CreER}(G2A) (A), Neurog1\textsuperscript{CreER}(G1C) (B), Neurod1\textsuperscript{CreER}(D1B) (C), and Neurod4\textsuperscript{CreER}(D4A) (D) drivers. The TM injection stages are indicated at the top. Magenta arrowheads 1 show cerebellar Purkinje stripes in (A), pontine nuclei in (B), and olive nuclei and other structures in the medulla in (D). Blue arrowheads 2 show the olfactory cortex and amygdala nuclei in (A) and (C), and the superior colliculus in (B). Brown arrowheads 3 show the inferior colliculus in (A), (C), and (D). Green arrowheads 4 show the area-specific distribution of superficial cortical neurons in (A), and cochlear nuclei in (B), (C), and (D). Scale bars, 1 mm.
| Region                                | Neurogenic-tagged stage | Driver line                          | Tagged territory          | Birthdate reported | Method       | Reference                          |
|--------------------------------------|-------------------------|--------------------------------------|---------------------------|--------------------|-------------|------------------------------------|
| Olfactory bulb                       | TM11.5–TM17.5           | Neurog2<sup>CreER</sup>(G2A)         | from deep to surface      | E10.5–E17.5        | [3H]thymidine | Hinds, 1968                        |
|                                      |                         | Neurod4<sup>CreER</sup>(D4A)         |                           |                    |             |                                    |
| Piriform cortex                      | TM11.5–TM14.5           | Neurog2<sup>CreER</sup>(G2A)         | E12.5 (peak)              | BrdU               | Sarma et al., 2011                  |
| Amygdala                             | TM12.5–TM15.5           | Neurog2<sup>CreER</sup>(G2A)         | distinct nuclei           | E11.5–E15.5        | [3H]thymidine | McConnell and Angevine, 1983       |
|                                      |                         | Neurod1<sup>CreER</sup>(D1B)         |                           |                    |             |                                    |
| Neocortex                            | TM11.5–TM12.5           | Neurod1<sup>CreER</sup>(D1B)         | from deep to surface      | E11.5–E12.5        | BrdU        | Hoerder-Suabedissen and Molnar, 2013|
| Subplate neuron (neocortex)          | TM11.5–TM12.5           | Neurod1<sup>CreER</sup>(D1B)         | E11.5–E12.5               | [3H]thymidine      | Caviness, 1982                        |
| Hippocampus pyramidal neuron         | TM13.5–TM17.5           | Neurog2<sup>CreER</sup>(G2A)         | from deep to surface      | E12.5–E16.5        | [3H]thymidine | Caviness, 1973                     |
|                                      |                         | Neurod1<sup>CreER</sup>(D1B)         |                           |                    |             |                                    |
|                                    |                          | Neurod4<sup>CreER</sup>(D4A)         |                           |                    |             |                                    |
| Habenula                             | TM11.5–TM16.5           | Neurog2<sup>CreER</sup>(G2A)         | from lateral to medial    | E11.5–E16.5        | [3H]thymidine | Angervine, 1970                    |
| Mammillary body                      | TM11.5–TM16.5           | Neurog2<sup>CreER</sup>(G2A)         | from lateral to medial    | E9.5–E13.5         | BrdU        | Szabo et al., 2015                  |
| Supramammillary nucleus              | TM10.5–TM16.5           | Neurog1<sup>CreER</sup>(G1C)         |                           |                    |             |                                    |
|                                      |                         | Neurod4<sup>CreER</sup>(D4A)         |                           | E10.5–E14.5        | [3H]thymidine | Shimada and Nakamura, 1973         |
| Superior colliculus                  | TM11.5–TM14.5           | Neurog1<sup>CreER</sup>(G1C)         | E11.5–E14.5               | [3H]thymidine      | Edwards et al., 1986                |
| Inferior colliculus                  | TM12.5–TM16.5           | Neurog2<sup>CreER</sup>(G2A)         | from lateral to medial    | E12.5–E15.5        | [3H]thymidine | Zeng et al., 2009                  |
|                                      |                         | Neurog1<sup>CreER</sup>(G1C)         |                           |                    |             |                                    |
|                                      |                         | Neurod1<sup>CreER</sup>(D1B)         |                           |                    |             |                                    |
|                                      |                         | Neurod4<sup>CreER</sup>(D4A)         |                           |                    |             |                                    |
| Cerebellum Purkinje cell             | TM10.5–TM12.5           | Neurog2<sup>CreER</sup>(G2A)         | sagittal stripes          | E10.5–E12.5        | BrdU        | Hashimoto and Mikoshiba, 2003      |
| Pontine nucleus                      | TM9.5–TM11.5            | Neurog1<sup>CreER</sup>(G1C)         | E12.5–E14.5               | BrdU               | Kawauchi et al., 2006               |
| Trapezioid body                      | TM11.5–TM12.5           | Neurog2<sup>CreER</sup>(G2A)         | E11.5–E12.5               | [3H]thymidine      | Pierce, 1973                          |
|                                      |                         | Neurog1<sup>CreER</sup>(G1C)         |                           |                    |             |                                    |
|                                      |                         | Neurod4<sup>CreER</sup>(D4A)         |                           |                    |             |                                    |
| Inferior olive                       | TM10.5–TM12.5           | Neurog2<sup>CreER</sup>(G2A)         | E9.5–E11.5                | [3H]thymidine      | Pierce, 1973                          |
| Cochlear nucleus                     | TM15.5–TM17.5           | Neurog1<sup>CreER</sup>(G1C)         | E14.5–E18.5 (second wave) | BrdU               | Shepard et al., 2019                |
|                                      |                         | Neurod1<sup>CreER</sup>(D1B)         |                           |                    |             |                                    |
|                                      |                         | Neurod4<sup>CreER</sup>(D4A)         |                           |                    |             |                                    |
| Spinal cord                          | TM9.5–TM13.5            | Neurog2<sup>CreER</sup>(G2A)         | from ventral to dorsal    | E9.5–E13.5         | [3H]thymidine | Nornes and Carry, 1978             |
|                                      |                         | Neurod4<sup>CreER</sup>(D4A)         |                           |                    |             |                                    |
| Dorsal root ganglion                 | TM9.5–TM12.5            | Neurod1<sup>CreER</sup>(D1B)         | E10.5–E13.5               | [3H]thymidine      | Lawson and Biscoe, 1979              |
|                                      |                         | Neurod4<sup>CreER</sup>(D4A)         |                           |                    |             |                                    |

Representative brain regions are tagged by the neurogenic tagging driver lines. The TM stages for tagging are compared with the birthdates determined by using nucleotide analogs in mice. The reported birthdates are adjusted to set the day of mating at E0.5.
the late TM17.5 stage (green arrowheads 4 in Figure 2A). Somatosensory and visual areas contained packed X-gal-labeled neurons, whereas frontal, motor, and medial cingulate areas contained less abundant labeled neurons. The biased areal distribution of these superficial neurons was also confirmed in neocortical sections (Figures 4F and 4F₀). A previous nucleotide-based neuronal birthdating study only reported a neurogenic gradient in the superficial layers (Bayer and Altman, 1991). Perhaps the discrete mosaic distribution of superficial neurons was more readily recognizable in a whole-mount cortical representation by using neurogenic tagging.

In the hippocampus, pyramidal neurons in the CA1–CA3 regions were labeled with embryonic TM injections (Figure 5A). Interestingly, deep and superficial neurons in the CA1 layer were separately labeled by different TM injection times (Figure 5A₀). They appear to be the neuronal subsets in the radial axis that have recently attracted much attention (Slomianka et al., 2011; Soltesz and Losonczy, 2018). The membrane-localized mGFP reporter, on the other hand, visualized the laminar organization of the hippocampus (Forster et al., 2006), where laminar-specific afferent axons from other brain regions and local projections of hippocampal proper neurons were differentially labeled depending on the TM stage (Figure 5B).

To characterize the timing of individual neurons that underwent TM-induced recombination in relation to their last cell cycle, we conducted a double injection of TM at the fixed TM12.5 stage and of EdU at a certain time point either before or after TM injection (Figures 5C–5F). Although the time courses for the emergence of double-positive neurons for EdU and the nls-bGal reporter varied across brain regions, in several brain domains such as the piriform cortex (Figure 5C), mammillary body (Figure 5D), and cerebellum (Figure 5E), TM-induced recombination was most prevalent in neurons 6–12 h after the final DNA synthesis. This time course was similar to that previously observed in olfactory bulb neurons (Hirata et al., 2019). There were, however, a few exceptions; for example, neurons in the trapezoid body were most frequently double labeled when EdU and TM were co-injected (Figure 5F), suggesting that TM-induced recombination occurs...
in the progenitor stage before the final DNA synthesis. Nonetheless, a peak of double-positive neurons was detected in a specific time interval between EdU and TM injections, implying that the TM-induced recombination marks the neurons only within a short differentiation time window (Figure 5F).

Neurog1CreER(G1C) driver using the neurog1 enhancer
The characteristic regions tagged by the Neurog1CreER(G1C) driver were the pontine nucleus (magenta arrowheads 1 in Figure 2B) and the superior colliculus in the midbrain (blue arrowheads 2 in Figure 2B), both of which were not labeled in whole-mount brain preparations by the other three drivers (Figures 2A–2D and Table 1).

In sections of the superior colliculus, neurons in the superficial sensory layers that receive retinal axons were labeled at TM12.5–TM13.5, and neurons in the deep motor layers were labeled sparsely for a more protracted TM11.5–TM14.5 (Figures S2A and S2B). This spatiotemporal pattern of labeling resembles the reported pattern of neurogenesis in the superior colliculus (Edwards et al., 1986), where superficial and deep-layer neurons are generated as distinct compartments following different time courses. Scattered neurons in the deep motor layers were also tagged by the other drivers at the same TM11.5–TM14.5 stages (Figures S2C and S2D), although they were invisible in the whole-mount preparations (Figure 2). The observation that the superficial layer neurons were only tagged by the Neurog1CreER(G1C) driver seems to be consistent with the idea that the superficial sensory layers and the deep motor layers of the superior colliculus are populated with neuronal populations of distinct origins.

Neurod1CreER(D1B) driver using the neurod1 enhancer
The Neurod1CreER(D1B) driver tagged only several externally visible structures in the whole-mount brains; some olfactory areas and the amygdala were significantly labeled at TM10.5–TM14.5 (blue arrowheads 2 in Figure 2C). The labeled amygdala nuclei overlapped with, but were distinct from, those labeled by the Neurog2CreER(G2A) driver (Figure 2A). In sections of the superior colliculus, brown arrowheads 3 in Figures 2A, 2C, and 2D and the cochlear nucleus (green arrowheads 4 in Figures 2B–2D) were labeled following the same spatiotemporal pattern shared
by the other drivers (Table 1). The late labeling of the cochlear nucleus after TM15.5 appeared to correspond with the second wave of neurogenesis in the dorsal cochlear nucleus reported recently (Shepard et al., 2019).

In cross-sections of the neocortex, a tangential band of neurons was labeled at the border between the neocortex and white matter at TM11.5–TM12.5 (Figure 6A). These cells appeared to be surviving subplate neurons in layer VIb (Friedlander and Torres-Reveron, 2009). The mGFP reporter selectively visualized their characteristic dense basal dendrites at the bottom of the cortical plate and widespread axons in layer 1 (Figure 6B; Clancy and Cauiller, 1999). Tagging at TM13.5–TM14.5 labeled only a few cortical neurons scattered in layers VI and V (Figure 6A). These neurons were mainly positioned in the medial cortex (Figures 6C and 6D), and their axons were observed to selectively project to the medial part of the striatum (Figures 6C and 6D), exhibiting a unique feature reported for neurons in the medial prefrontal area (Gerfen et al., 2013; Mailly et al., 2013). Later-stage TM injections did not label neurons in the upper layers (Figure 6A), showing a clear contrast to the inside-out pattern throughout the cortical plate induced by the Neurog2CreER(G2A) driver (Figure 4A).

**Neurod4CreER(D4A) driver using the neurod4 enhancer**

The Neurod4CreER(D4A) driver abundantly tagged neurons in the caudal parts of the brain such as the medulla and the spinal cord (magenta arrowheads 1 in Figure 2D). The spatial pattern and time course of labeling in these caudal regions resembled those of Neurog2CreER(G2A) (Figure 2A and Table 1), consistent with the idea that neurod4 is a downstream effector of neurog2 (Seo et al., 2007; Masserdotti et al., 2015). However, there
were differences between the two drivers; for example, the neocortex and cerebellar Purkinje cells were not significantly labeled by the Neurod4CreER(D4A) driver (Figure 2D and Table 1). This driver notably visualized long projecting axons in several brain regions. For example, mGFP-labeled optic axons from the retinal ganglion cells tagged at TM13.5–TM14.5 were targeted at the superficial layers of the superior colliculus (Figure S2D). The habenular neurons in the thalamus were generated in a lateral-to-medial gradient (Figure 7A) (Angevine, 1970; Aizawa et al., 2007), whereas their axons exhibited a periphery-to-center arrangement within the fasciculus retroflexus, which is the axon tract formed by habenular neurons (Figure 7B).

Using this driver, we also characterized the cell-cycle timing of neurons that underwent TM-induced recombination. TM injection at the fixed E14.5 stage labeled neurons in the hippocampal CA3 region (Figure 7C), habenula (Figure 7D), supramammillary nucleus (Figure 7E), and inferior colliculus (Figure 7F). The time-spaced injection of EdU indicated that neurons were most susceptible to TM-induced recombination at 6–12 h after the final DNA synthesis. Although the time course of double labeling seemed slightly delayed compared with that of Neurog2CreER(G2A) (Figures 5C–5F), such a comparison might not be meaningful, as different neurons and TM stages were involved and we could not quantitatively determine the time lag in the gene expression cascade from neurog2 to neurod4.

DISCUSSION

Neurogenic tagging resource

In the past few decades, various important discoveries have been made through neuronal birthdating (Bayer and Altman, 1987; Govindan et al., 2018). The neurogenic tagging resource presented such as optogenetics and chemogenetics (Deisseroth et al., 2006; Alexander et al., 2009), which enable the functional manipulation of birthdate-classified neuronal subsets. We hope that the unique ideas of researchers will lead to important discoveries using this resource, given that the neuronal birthdate is so fundamental to organizing the neural circuitry.

This neurogenic tagging method uses the expression timing of neuronal differentiation genes; therefore, the underlying biological principle is significantly different from that of the traditional method using nucleotide analogs. The four neuronal differentiation genes exploited in our resources were selected on the basis of their seemingly transient expression during the maturation phase of neurons as shown in a previous study (Mattar et al., 2008) and databases (Allen Developmental Mouse Brain Atlas, https://developingmouse.brain-map.org; GENSAT: Gene Expression Nervous System Atlas, http://www.gensat.org/index.html). As expected, the neurons underwent loxP recombination 6–12 h after the final DNA synthesis in different parts of the nervous system (Figures 5C–5E and 7C–7F), and recapitulated the patterns consistent with those seen in birthdating analyses using nucleotide analogs (Table 1). In some exceptional cases, such as the trapezoid body (Figure 5F), TM-induced recombination seemed to occur within a short time window during the progenitor stage before the final DNA synthesis. In the pontine nucleus and the dorsal root ganglion (Table 1), the earlier tagging stages, compared with the previously determined birthdates, also suggest that TM-induced recombination occurs pre-mitotically in these regions. Thus, neurogenic tagging using neuronal differentiation genes appears to mark the timing of neuron commitment rather than cell-cycle exit (Hirata et al., 2019). Recently, increasing evidence has indicated that neuron commitment and cell-cycle exit are dissociable processes in this study resolves some of the limitations of the previous method and forges a link between this traditional histological classification and the new experimental approaches and applications. Specifically, neurogenic tagging can be easily combined with various molecular genetic tools.
A prime example is the cortical basal progenitor, which is the fate-restricted neural progenitor that undergoes additional cell divisions but only generates neurons (Lodato and Arlotta, 2015; Hevner, 2019). Our ongoing study shows that these cortical basal progenitors are indeed tagged by one of the neurogenic tagging driver lines.

During development, the neurod1 gene is widely expressed in many nervous systems (Miyata et al., 1999; Mattar et al., 2008), and a short enhancer element of this gene is commonly used to study neuronal differentiation in vivo and in vitro (Guerrier et al., 2009). Somewhat contradictory to the general use of this gene, the Neurod1CreER(D1B) driver tagged only a restricted subset of neurons (Figures 2Ca and 6). This might be because we obtained only one mouse line that exhibited significant CreER activity after the intensive production of transgenic mice by using the neurod1 gene. Regardless, the unique characteristics of neuronal tagging by the Neurod1CreER(D1B) driver shown in this study and NeuroGT demonstrate the usefulness of this driver line for some specific purposes.

The present collection of four neurogenic tagging drivers does not fully cover all brain regions. For example, the majority of neurons in the striatum and hypothalamus were unlabeled by any of the drivers. To complement the current collection a promising candidate is the Ascl1 gene, which is expressed in the basal plate of the neural tube in a manner complementary to that of neuronal differentiation genes used in this study (Ma et al., 1997; Wilkinson et al., 2013). Furthermore, the mice that express CreER under the Ascl1 gene enhancer have been reported to apparently show birthdate-dependent recombination (Tg(Ascl1-cre/Esr1*)1Jejo, MGI: 3767428, Battiste et al., 2007; Ascl1tm1.1(cre/ERT2)Jejo, MGI: 4452601, Kim et al., 2008; Sudarov et al., 2011). In the future, we hope to include detailed image data of neurons tagged by Ascl1CreER mice in the NeuroGT database.

To date, multiple CreER-expressing mice under the control of neuronal differentiation genes have been generated by several groups (Neurog2tm1(cre/Esr1*)And, MGI: 2652037, Zirlinger et al., 2002; Tg(Neurog1-cre/ERT2)1Good/J, MGI: 3807088, Koundakjian et al., 2007; Neurog2iCreERT2, Winpenny et al., 2011; Neurog2tm1(cre/ERT2)Ggc, MGI: 5431772, Florio et al., 2012; Tg(Neurod1-cre/ERT2)M1Fcal, MGI: 5582823, Aprea et al., 2014). Although these mice have been primarily used for cell lineage analyses related to the manipulated genes, they are theoretically applicable to birthdate-based classification and manipulation of neurons. Our Nerog2CreER(G2A) driver mouse generated by BAC transgenesis has a greater CreER activity than that under the endogenous locus in Neurog2-CreER knockin mice (Zirlinger et al., 2002). However, suitable drivers differ depending on the research purpose. It is important to note that information in the NeuroGT database can be useful even for developing a research design that does not use our mouse lines.

Features highlighted by the present neurogenic tagging analyses
Our original motivation for developing the neurogenic tagging method was to visualize axon projections of birthdate-classified
neuron subsets in the olfactory bulb in adulthood. This method was indeed effective in revealing the birthdate-dependent olfactory axon trajectories (Hirata et al., 2019). It has also been applied to examine birthdate-dependent compartments in the cerebellum (Tran-Anh et al., 2020; Zhang et al., 2020). The present analyses using the multiple neurogenic tagging drivers added interesting findings, three of which are discussed below.

**Corpus callosum**

Within the corpus callosum, early-born (TM12.5–TM13.5) and late-born (TM16.5–TM17.5) cortical neurons formed segregated axon bundles (Figures 4B and S1D–S1G), consistent with the current knowledge that the callosal projections are formed by a subset of layers II/III and V neurons in the neocortex (Fame et al., 2011). The segregated construction of the callosum by pioneering and following axons has been proposed (Koester and O’Leary, 1994; Rash and Richards, 2001). Although the previous studies have considered the time lag of axon arrivals from the medial and lateral cortical areas (Piper et al., 2009; Nishikimi et al., 2011; Zhou et al., 2013), the present analysis indicates that the time lag between the early-born and late-born callosal neurons in different layers also underlies the organization of this axon bundle, which is the largest in placental mammals (Suarez et al., 2014).

**Fasciculus retroflexus**

Neurogenic tagging revealed a periphery-to-center axon topology within the fasciculus retroflexus (Figures 7A and 7B). A previous axon-tracing analysis reported that the medial and lateral habenula nuclei project their axons into the core and shell of this tract, respectively (Herkenham and Nauta, 1979). To explain this organization, the present study provides a hint: late-growing axons might penetrate the center of the bundle pre-formed by early-growing axons. In general, for axon tracts positioned on the brain surface, late-growing axons are sequentially added to the pial superficial space, displacing the pre-existing axons deeper (Walsh et al., 1983; Walsh and Guillery, 1985; Inaki et al., 2004; Yamatani et al., 2004). Although little is known about the development of internally located axon tracts, new axons are found to grow in the center of pre-formed axon bundle in the mushroom body of *Drosophila* (Kurusu et al., 2002). Within the optic nerve, which is an isolated axon bundle, newly growing axons are positioned near the center (Walsh, 1986).

**Hippocampus pyramidal neurons**

Although hippocampal neurons in the single pyramidal layer have been regarded as a homogeneous population, recent studies indicate that hippocampal pyramidal neurons are in fact heterogeneous along the radial axis (Slomianka et al., 2011; Soltesz and Losonczy, 2018). The deep and superficial subsets in the layer express different genes, make different connections, and are expected to have distinct functions in different behavioral contexts (Mizuseki et al., 2011; Lee et al., 2014; Danielson et al., 2016). These subsets are assumed to differentiate at different times in an inside-out pattern (Slomianka et al., 2011; Soltesz and Losonczy, 2018). The present neurogenic tagging analysis confirmed this assumption and allowed effective classification of these neuronal subsets (Figures 5A and 5A’). An advantage of this neurogenic tagging is that this classification can now be connected to functional tests. We hope that the neurogenic tagging resource will benefit ingenious ideas that will lead to future scientific discoveries.

**Limitations of the study**

Neurogenic tagging is different from nucleotide-based neuronal birthdating. The timing of recombination in the cell cycle differs among neurons and appears to take place prior to the final mitosis in some neurons. It is important to consider this difference when the neurogenic tagging approach is taken, as this difference might be beneficial in some cases. In the NeuroGT database, the anatomical ontology of tagged brain regions is not yet available but will be included in the future to enable searching by anatomical regions.

**STAR★METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.crmeth.2021.100012.

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**AUTHOR CONTRIBUTIONS**

T.H. conceived the research, performed the experiments, analyzed the data, and wrote the manuscript with help from other authors. G.S. and H.K. established the mouse lines. S.O. and T.F. converted the images of neurogenic-tagged serial sections into high-resolution digital data. Y.T., H.I., and S.O. processed the image data and constructed the NeuroGT database.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.
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## Key Resources Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Chicken anti-β-GAL  | Abcam  | Cat# ab9361; RRID: AB_307210 |
| Rabbit anti-GFP     | MBL International | Cat# 598; RRID: AB_591816 |
| Chicken anti-GFP antibody | Abcam | Cat# ab13970; RRID: AB_300798 |
| Rat monoclonal anti-CTIP2 antibody | Abcam | Cat# ab51502; RRID: AB_882455 |
| Mouse monoclonal anti-SATB2 antibody | Abcam | Cat# ab51502; RRID: AB_882455 |
| Rabbit anti-GABA antibody | Sigma-Aldrich | Cat# A2052; RRID: AB_477652 |
| Rabbit anti-NRP1 antibody | Kawakami et al., 1996 | |
| Donkey biotin-SP-conjugated anti-rabbit IgG | Jackson Immunoresearch | Cat# 711-065-152; RRID:AB_2340593 |
| Donkey Alexa488-conjugated anti-rabbit IgG | Life Technologies | Cat# A-21206; RRID: AB_141708 |
| Donkey Cy3-conjugated anti-rabbit IgG | Jackson Immunoresearch | Cat# 711-165-152; RRID: AB_2307443 |
| Donkey Alexa488-conjugated anti-chicken IgY | Jackson Immunoresearch | Cat# 703-545-155; RRID: AB_2340375 |
| Donkey biotin-SP-conjugated anti-chicken IgY | Jackson Immunoresearch | Cat# 703-065-155; RRID: AB_2313596 |
| Donkey Cy3-conjugated anti-rat IgG | Jackson Immunoresearch | Cat# 712-166-153; RRID: AB_2340669 |
| Donkey Cy3-conjugated anti-mouse IgG | Jackson Immunoresearch | Cat# 715-165-150; RRID: AB_2340813 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Tamoxifen           | Sigma-Aldrich | T5648; CAS: 10540-29-1 |
| Corn oil            | Sigma-Aldrich | C8267; CAS: 8001-30-7 |
| Progesterone        | Fujifilm Wako | 161-14531; CAS: 57-83-0 |
| 5-ethynyl-2′-deoxyuridine (EdU) | Tokyo Chemical Industry | E1057; CAS: 61135-33-9 |
| Alexa555 azide triethylammonium salt | Thermo Fisher Scientific | A20012 |
| X-gal               | Fujifilm Wako | 029-15043; CAS: 7240-90-6 |
| Elite ABC kit       | Vectastain | PK-6100; RRID: AB_2336819 |
| DAB                 | Dojindo | 347-00904; CAS: 7411-49-5 |
| **Deposited data** |        |            |
| NeuroGT database    | This paper | https://ssbd.riken.jp/neurogt/ |
| Raw images for NeuroGT database and all figures, and counts of EdU-positive and -negative neurons | This paper | SSBD:repository: https://doi.org/10.24631/ssbd.repos.2021.03.001 |
| **Experimental models: organisms/strains** |        |            |
| Mouse: C57BL/6       | Japan SLC | RRID: MGI:5295404 |
| Mouse: ICR           | Japan SLC | RRID: MGI:5462094 |
| Mouse: Neurog2<sup>C1wER</sup>(G2A); C57BL/6-Tg(Neurog2-cre/ERT2)G2ATahi | Hirata et al., 2019 | Accession #: CDB0512T-1: http://www2.clst.riken.jp/arg/TG%20mutant%20mice%20list.html |
| Mouse: Neurog1<sup>C1wER</sup>(G1C); C57BL/6-Tg(Neurog1-cre/ERT2)G1CTahi | This paper | Accession #: CDB0526T: http://www2.clst.riken.jp/arg/TG%20mutant%20mice%20list.html |
| Mouse: Neurod1<sup>C1wER</sup>(D1B); C57BL/6-Tg(Neurod1-cre/ERT2)D1BTahi | This paper | Accession #: CDB0510T: http://www2.clst.riken.jp/arg/TG%20mutant%20mice%20list.html |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to Tatsumi Hirata (tathirat@nig.ac.jp).

**Materials availability**
The neurogenic tagging mouse lines are deposited at RIKEN BDR (http://www2.clst.riken.jp/arg/TG%20mutant%20mice%20list.html) and provided with a completed materials transfer agreement. The accession numbers are as follows: Neurog2\textsuperscript{CreER(G2A)} driver, CDB0511T; Neurog1\textsuperscript{CreER(G1C)} driver, CDB0526T; Neurod1\textsuperscript{CreER(D1B)} driver, CDB0510T; Neurod4\textsuperscript{CreER(D4A)} driver, CDB0511T. All other reagents generated in this study are available from the Lead Contact.

**Data and code availability**
All images acquired in this study are available in the NeuroGT database at https://ssbd.riken.jp/neurogt/. Raw images and unprocessed data related to this study and NeuroGT are archived in SSBD:repository (https://doi.org/10.24631/ssbd.repos.2021.03.001).

### Table: REAGENT or RESOURCE SOURCE IDENTIFIER

| RESOURCE or RESOURCE | SOURCE | IDENTIFIER |
|----------------------|--------|------------|
| Mouse: Neurod4\textsuperscript{CreER(D4A)}; C57BL/6-Tg(Neurod4-cre/ERT2) D4ATahi | This paper | Accession # CDB0511T: http://www2.clst.riken.jp/arg/TG%20mutant%20mice%20list.html |
| Mouse: Tau\textsuperscript{inGFP-LacZ}; 129P2-Map\textsuperscript{tm2Arbr} | Hippenmeyer et al., 2005 | RRID: IMSR_JAX:021162 |
| Mouse: ROSA26R-Cre; Gt(Rosa)26Sor\textsuperscript{tm1Sor} | Soriano, 1999 | MGI: 1861932 |
| Oligonucleotides | | |
| Cre primers1: 5’-TAAAGATATCTCAGTA CGACGGTG-3’ | Hirata et al., 2019 | N/A |
| Cre primers2: 5’-TCTCTGACCAG TAGCCTTAGC-3’ | Hirata et al., 2019 | N/A |
| Recombinant DNA | | |
| BAC clone RP24-347K19 | BACPAC Resource Center | RP24-347K19 |
| BAC clone RP23-280C11 | BACPAC Resource Center | RP23-280C11 |
| BAC clone RP23-55O18 | BACPAC Resource Center | RP23-55O18 |
| Cre\textsuperscript{ERT2} | Feil et al., 1997 | N/A |
| p23loxZeo | Dr. Junji Takeda, Osaka University | N/A |
| p24loxZeo | Dr. Junji Takeda, Osaka University | N/A |
| Oligonucleotides | | |
| Cre primers1: 5’-TAAAGATATCTCAGTA CGACGGTG-3’ | Hirata et al., 2019 | N/A |
| Cre primers2: 5’-TCTCTGACCAG TAGCCTTAGC-3’ | Hirata et al., 2019 | N/A |
| Recombinant DNA | | |
| BAC clone RP24-347K19 | BACPAC Resource Center | RP24-347K19 |
| BAC clone RP23-280C11 | BACPAC Resource Center | RP23-280C11 |
| BAC clone RP23-55O18 | BACPAC Resource Center | RP23-55O18 |
| Cre\textsuperscript{ERT2} | Feil et al., 1997 | N/A |
| p23loxZeo | Dr. Junji Takeda, Osaka University | N/A |
| p24loxZeo | Dr. Junji Takeda, Osaka University | N/A |
| Software and algorithms | | |
| Cell Sens Standard (ver.1.16) | Olympus | N/A |
| Fluoview FV-ASW (ver 4.02) | Olympus | N/A |
| SCN400 Client (version 2.2.0.3789) | Leica | N/A |
| Photoshop CC 2021 (22.2.0) | Adobe | N/A |
| Fiji / ImageJ (2.0.0) | Schindelin et al., 2012 | RRID: SCR_002285 |
| Microsoft Excel (Mac 2019, 16.46) | Microsoft | N/A |
| GraphPad Prism 8 | GraphPad | N/A |
| Other | | |
| Slide scanner (SCN400) | Leica | N/A |
**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Mice**
C57BL/6 wild-type mice (RRID: MGI: 5295404) and outbred ICR foster mothers (RRID: MGI: 5462094) were purchased from Japan SLIC Inc. The Tau$^{mGFP-nLacZ}$ reporter mice, officially known as 129P2-Mapt$^{tm2Arb}$ (RRID: IMSR_JAX: 021162, Hippenmeyer et al., 2005), with a CD-1 mixed background were provided by Dr. Silvia Arber at Friedrich Miescher Institute for Biomedical Research and backcrossed with C57BL/6 wild-type mice for at least four generations before use in this study. All mice were maintained in the animal facility of the National Institute of Genetics or RIKEN BDR. They were group housed in conventional cages under controlled conditions (temperature 23 ± 2°C, humidity 50 ± 10%, 12 h light-dark cycle), and food and water were provided ad libitum. All procedures for the care and treatment of mice were approved by the Institutional Animal Committees and carried out according to their guidelines.

Heterozygous neurogenic tagging driver mice were mated with homozygous reporter mice. The day on which a vaginal plug was detected and the day of birth were designated as embryonic day 0.5 (E0.5) and postnatal day 0 (P0), respectively. TM treatment was performed by intraperitoneally injecting a staged pregnant mouse with 250 μL of corn oil (C8267, CAS#8001-30-7, Sigma-Aldrich) containing 9 mM tamoxifen (T5648, CAS#10540-29-1, Sigma-Aldrich) and 5 mM progesterone (161-145-31, CAS# 57-83-0, Fujifilm Wako). As TM often delays delivery, when pups were not born by E19.5, they were collected by caesarian delivery and given to ICR foster mothers to bring them to full term, as described in the previous study (Hirata et al., 2019).

**METHODS DETAILS**

**Generation of neurogenic tagging mouse lines**
The NeurogCreER$^{G2A}$ line, officially named C57BL/6-Tg(Neurog2-cre/ERT2)G2ATahi, was generated using the genomic BAC clone RP24-347K19 encoding the mouse neurog1, which was obtained from the BACPAC Resource Center (Children’s Hospital Oakland Research Institute, Oakland, CA). The entire coding sequence of exon1 was replaced by CreER(T2) (Feil et al., 1997) (gifted by Dr. Pierre Chambon), and the loxP site in the vector backbone (pTARBAC) was deleted by replacement with the zeocin resistance gene in the p24loxZeo (gifted by Dr. Junji Takeda). For the Neurod1CreER(D1B) line, officially named C57BL/6-Tg(Neurod1-cre/ERT2)D1BTahi, the CreER(T2) coding sequence was inserted at the ATG sequence in the BAC clone RP23-280C11 encoding the mouse neurod1 (BACPAC Resource Center). For the Neurod4CreER(D4A) line, officially named C57BL/6-Tg(Neurod4-cre/ERT2)D4ATahi, the CreER(T2) coding sequence was inserted at the ATG sequence in the BAC clone RP23-55018 encoding the mouse neurod4 (BACPAC Resource Center), and the loxP site in the vector backbone (pBACe3.6) was deleted by replacement with the zeocin resistance gene in the p23loxZeo (gifted Dr. Junji Takeda). Except for the above-mentioned wild-type loxP sequences, the BAC vector backbone sequences, including a few genes, were unmodified.

The constructed BAC recombinants were injected into fertilized eggs with a C57BL/6 background, and the tail genomic DNA of the resulting mice was assayed by PCR for the integration of the transgene. Transgene containment was determined by PCR using internal Cre recombinase primers 5’-TAAGATATCTCAGTGACGGTTG-3’ and 5’-TCTCTGACCAGAGTCATCCTAGC-3’, resulting in the amplification of 300-bp fragments. In total, eleven, two, and five mice were found to have random integrations of the transgene for neurog1, neurod1, and neurod4, respectively, including multiple BAC constructs for each gene. These mouse lines were assayed for CreER activity by crossing them with ROSA26R Cre reporter mice, officially named Gt(ROSA)26Sortm1Sor (RRID: MGI: 1861932, Soriano, 1999). After crossing, pregnant mice were injected with TM solution at E12.5 or E14.5. Embryos were dissected from the dam at E18.5-E19.5, and their isolated brains were whole-mount stained with X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) to detect β-galactosidase activity from Cre recombinase in the reporter line. Histochemistry

The mice were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS). For whole-mount visualization of the nls-β-GAL signals, the brains were immediately dissected and immersed in PBS containing 1 mg/mL of X-gal, 5 mM K3[Fe(CN)6], 5 mM K4[Fe(CN)6], 2 mM MgCl2, and 1% Tween 20 for 2.5 hours at 37°C (Saga et al., 1992). To prepare cryosections, the dissected brains were further fixed with 4% PFA/PBS for 8–24 hours, immersed in 30% sucrose/PBS, and frozen in a 2:1 mixture of OCT compound (Sakura Finetek) and 30% sucrose/PBS. Coronal sections of 20-μm thickness were cut on a cryostat, and mounted on MAS-coated glass slides (Matsunami Glass).

The sections were washed with 10 mM Tris-HCl (pH 7.4), 130 mM NaCl and 0.1% Tween 20 (TBST) and incubated with rabbit anti-GFP antibody (1:1000, #598, MBL International Cat# 598, RRID: AB_591816) diluted in PBS containing 0.1% Tween 20 and 0.5% BSA. The sections were then incubated in the primary antibody solution overnight at 4°C. After washing, the sections were incubated with a 1:1000 GFP antibody (1:1000, #598, MBL International Cat# 598, RRID: AB_591816) diluted in PBS containing 0.1% Tween 20 and 0.5% BSA. The sections were then washed and incubated with secondary antibody (goat anti-rabbit) for 1 hour at room temperature. After washing, the sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole) and mounted on coverslips with Aqua_poly (Matsunami Glass).
blocking reagent (FP1020, PerkinElmer) overnight at 4°C. After treatment with 0.7% H2O2 in methanol for 5 min at 4°C, antibody labeling was detected using donkey biotin-SP-conjugated anti-rabbit IgG antibody (1:1000, Jackson Immunoresearch Cat# 711-065-152. RRID: AB_2340593), amplified with the Elite ABC kit (1:300, PK-6100, Vectastain, RRID: AB_2336819), and visualized in TBST containing 0.13 mg/mL 3,3′-diaminobenzidine tetrahydrochloride (DAB, 347-00904, CAS#7411-49-5, Dojindo) and 0.03% H2O2. In some specimens, the binding of anti-GFP antibodies was fluorescently visualized with donkey Alexa488-conjugated anti-rabbit IgG (1:1000, Life Technologies Cat# A-21206, RRID: AB_141708). For antibody staining of nls-β-GAL, the sections were heat-treated in an antigen retrieval solution (HistoVT One, Nacalai Tesque) by autoclaving at 105°C for 2 min and incubated with chicken anti-β-GAL antibody (1:2000, Abcam Cat# ab9361, RRID: AB_307210) diluted in PBS containing 0.1% Tween 20 and 0.5% blocking reagent overnight at 4°C. The bound antibodies were detected with donkey Alexa488-conjugated anti-chicken IgY (1:1000, Jackson Immunoresearch Cat# 703-545-155, RRID: AB_2340375). In NeuroGT database, the binding of anti-β-GAL antibodies was enzymatically visualized with DAB. For that, the sections were treated with 0.7% H2O2/methanol for 5 min at 4°C after antigen retrieval and incubated with the anti-β-GAL primary antibody, donkey biotin-SP-conjugated anti-chicken IgY (1:1000, Jackson Immunoresearch Cat# 703-065-155, RRID: AB_2313596), and the Elite ABC kit, as described above.

For the double immunostaining of cortical neurons (Figures S1A and S1B), antigen-retrieved sections were incubated with chicken anti-β-GAL antibody together with rat monoclonal anti-CTIP2 (1:1000, Abcam, Cat# ab18465, RRID: AB_2064130) or mouse monoclonal anti-SATB2 (1:1000, Abcam, Cat# ab51502, RRID: AB_882455) antibody, and then stained with donkey Alexa488-conjugated anti-chicken IgY and donkey Cy3-conjugated anti-rat IgG (1:1000, Jackson Immunoresearch Cat# 715-165-150, RRID:AB_2340669) or donkey Cy3-conjugated anti-mouse IgG (1:1000, Jackson Immunoresearch Cat# 715-165-150, RRID:AB_2340813) antibodies. In Figure S1C, to minimize cross-reactions of the anti-rabbit secondary antibody, sections were first stained with rabbit anti-GABA (1:2000, Sigma-Aldrich, Cat# A2052, RRID: AB_477652) and donkey Cy3-conjugated anti-rabbit IgG (1:1000, Jackson Immunoresearch Cat# 711-165-152, RRID: AB_2307443) antibodies, and then visualized for β-GAL as described. Rabbit anti-NRP1 antibody was prepared and used as previously described (1:2000, Kawakami et al., 1996; Sato et al., 1998). In Figures S1E and S1G, sections were immunostained for mGFP with a chicken anti-GFP (1:1000; Abcam Cat# ab13970, RRID: AB_300798) and donkey Cy3-conjugated anti-rabbit IgG (1:1000, Jackson Immunoresearch Cat# 711-165-152, RRID: AB_2307443) antibodies, and then visualized for β-GAL as described above. The fluorescent samples were counterstained with DAPI (4’,6-diamidino-2-phenylindole, 045-30361, CAS#28718-90-3, Fujifilm Wako).

**Image acquisition**

Whole-mount brain images (Figure 2) were captured with a digital camera (EOS 6D, Canon) mounted on a dissection microscope (SZ61, Olympus). The sections (Figures 4A, 4B, 5A, 5B, 6A, 7A, 7B, S2A, and S2C) were imaged with a CCD camera (Olympus DP71) attached to a conventional fluorescent microscope (Zeiss Axioplan2) using Cell Sens Standard software. Wide tile images were generated using the Prism8 software (GraphPad).

**EdU incorporation assay**

A thymidine analog, 5-ethyl-2′-deoxyuridine (EdU, Tokyo Chemical Industry) was intraperitoneally injected into a pregnant mouse (50 mg/kg body weight) at the indicated time points before or after TM injection. The brains were dissected from the mice at P0, and coronal sections of 16-μm thickness were prepared as explained above. The sections were antigen-retrieved as described above and immunostained with chicken anti-β-GAL and donkey Alexa488-conjugated anti-chicken IgY antibodies. Subsequently, the incorporated EdU was detected in a solution of 0.1 M Tris (pH 7.6), 2 mM CuSO4, 3 μM Alexa555 azide triethylylammonium salt (A20012, Thermo Fisher Scientific), and 10 mM ascorbic acid for 40 min at room temperature. The numbers of neurons labeled for β-GAL and those doubly labeled for β-GAL and EdU were manually counted for each mouse with a 40× objective lens (Plan-Apochromat) under a fluorescent microscope (Zeiss Axiosplan2) with 38 HE (ex470/40, em525/50) and 15 (ex549/12, em590) filter sets. The exact numbers of animals and neurons used for quantification are shown in the figures and legends. The scattered plots (Figures 5C–5F and 7C–7F) were generated using the Prism8 software (GraphPad).

**Construction of the NeuroGT database**

The brains were collected from P7 mice that had been tagged on a single day during E9.5–E18.5 using each neurogenic tagging mouse driver. Whole brains were cut into serial coronal sections (20-μm thick) from anterior to posterior, and each section was pasted on a batch of 10 glass slides in rotation. From the batch, three interspaced slides were antibody stained for the mGFP reporter, and three others were antibody stained for the nls-β-GAL reporter. The antibody reactions were enzymatically visualized using DAB, as described above. Slide-fixed sections were dehydrated and mounted using Entellan New (Merck). The staining procedures and antibodies were slightly modified for the samples, and all these details are provided as meta-information in the NeuroGT database.
Images of the glass slides containing the immunostained multiple sections were acquired with a slide scanner (SCN400, Leica) using its designated software (Leica SCN400 Client Version 2.2.0.3789). The contrast and brightness were adjusted automatically. The images acquired using a 20× objective lens were extracted and separated into individual images of single sections using the Fiji distribution package of ImageJ (RRID: SCR_002285; Schindelin et al., 2012). The images of individual sections were aligned along the antero-posterior axis of the brain by rotating at 180° if needed.

The NeuroGT database was established as a web-based system using the Django framework with the PostgreSQL database on a Linux container with the Nginx server. An interactive image viewer was constructed using JavaScript. To encourage the operation, the database was organized as SSBD (Tohsato et al., 2016) with common meta-information such as organism name and contact information of the corresponding author in a unified format. The meta-information specific to NeuroGT, including driver name, TM stage and reporter, has also been provided.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

The exact numbers of animals and neurons used for quantification are shown in Figures 5C–5F and 7C–7F. The proportions of tagged neurons containing EdU were calculated using Excel for Mac (Microsoft) and the raw counting data were archived in SSBD:repository (https://doi.org/10.24631/ssbd.repos.2021.03.001). The scattered plots and means were generated using the GraphPad Prism 8. In this study, the purpose of quantification was to show a trend of the continuous developmental process but not to differentiate a specific developmental stage from the others; thus, dichotomous significance testing was not performed.

**ADDITIONAL RESOURCES**

All images acquired in this study are available in the NeuroGT database at https://ssbd.riken.jp/neurogt/. Raw images and other data are available in SSBD:repository (https://doi.org/10.24631/ssbd.repos.2021.03.001).