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RESEARCH REPORT

STEM CELLS AND REGENERATION

Drosophila intermediate neural progenitors produce lineage-dependent related series of diverse neurons

Yu-Chun Wang[1,*], Jacob S. Yang[1,*], Rebecca Johnston[1], Qingzhong Ren[1], Ying-Jou Lee[1], Haojiang Luan[1], Thomas Brody[2], Ward F. Odenwald[2] and Tzumin Lee[1,‡]

ABSTRACT

Drosophila type II neuroblasts (NBs), like mammalian neural stem cells, deposit neurons through intermediate neural progenitors (INPs) that can each produce a series of neurons. Both type II NBs and INPs exhibit age-dependent expression of various transcription factors, potentially specifying an array of diverse neurons by combinatorial temporal patterning. Not knowing which mature neurons are made by specific INPs, however, conceals the actual variety of neuron types and limits further molecular studies. Here we mapped neurons derived from specific type II NB lineages and found that sibling INPs produced a morphologically similar but temporally regulated series of distinct neuron types. This suggests a common fate diversification program operating within each INP that is modulated by NB age to generate slightly different sets of diverse neurons based on the INP birth order. Analogous mechanisms might underlie the expansion of neuron diversity via INPs in mammalian brain.

KEY WORDS: *Drosophila* type II neuroblasts, Adult brain, Cell lineage analysis, Intermediate neural progenitors, Neuronal cell fate, Temporal identity

INTRODUCTION

Expansion of mammalian neocortex involves neurogenesis via intermediate neural progenitors (INPs). One INP can undergo multiple rounds of asymmetric cell division to deposit a series of neural precursors (Lui et al., 2011). A growing body of evidence suggests that diverse neurons are specified based on their precursor identity (Franco et al., 2012; Molyneaux et al., 2007; Wonders and Anderson, 2006). To track specific neural progenitors becomes essential for understanding brain development.

In *Drosophila melanogaster*, neurons of the supraesophageal ganglion (the fly cerebrum) originate from ~100 neuroblasts (NBs) per hemisphere (Urbach et al., 2003). Most NBs yield a series of ganglion mother cells (GMCs) that divide once to make related pairs of distinct neurons, governed by birth order and Notch-mediated binary A/B fate decision (Lin and Lee, 2012). In 2008, three groups independently reported eight mammalian type neuronal lineages (referred to as type II lineages) in the developing *Drosophila* larval brain (Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). One type II NB can bud off multiple INPs that can each yield several GMCs to deposit ~10 neurons following each self-renewing NB division. The developing type II NB clones thus carry many more neurons than conventional type I NB clones. They further exhibit extraordinarily complex neurite tracts, implicating exceptional progeny diversity (Izergina et al., 2009). Notably, both type II NBs and INPs exhibit age-dependent expression of various transcription factors, the combinatorial expression of which may specify distinct offspring fates based on the birth order of INPs as well as their derived GMCs (Bayraktar and Doe, 2013). Besides sharing common proliferation patterns, the *Drosophila* type II NB lineages mimic many aspects of the less genetically tractable mammalian neural stem cell lineages. Most, if not all, type II NBs produce significant numbers of glia (Viktorin et al., 2011). In addition, apoptosis plays an important role in the shaping of the final pools of type II NB offspring (Jiang and Reichert, 2012). However, the characterization of type II lineages has been largely restricted to larval development prior to neural circuit formation, providing minimal insight into the mature identity of the derived progenies.

Here we mapped type II NB lineages in the adult *Drosophila* brain by targeted clonal labeling. We learn that neurons made by a single INP exhibit diverse neurite trajectories indicative of distinct neuronal classes. The discrete neuronal classes arise in an invariant sequence from the serially derived GMCs made by a common INP. In contrast to the production of distinct GMCs by a single INP, one type II NB yields a series of analogous INPs that each generates a specific but related sequence of diverse neurons. These observations implicate distinct mechanisms of temporal fate diversification acting in parallel along the sequence of INPs and the sequence of the progeny of each INP to specify arrays of diverse neurons in INP-containing neuronal lineages.

RESULTS AND DISCUSSION

Mapping type II NB lineages by targeted clonal analysis

Twin-spot MARCM allows differential labeling and thus independent tracking of sister clones derived from a common precursor for enhanced cell lineage analysis (Yu et al., 2009). It has been applied to resolve a protracted heterogeneous type I NB lineage through the identification of relevant clones with lineage-characteristic morphologies from thousands of mosaic brains (Lin et al., 2012). In such non-selective clonal analysis, the labeled cerebral clones could arise from any of the ~100 NB lineages per hemisphere. Given the anticipated extraordinary complexities in both proliferation patterns and clone morphologies of type II NB lineages, it would be extremely challenging to isolate clean type II lineage-derived clones and decipher their lineage relationships from sample brains with complex, arbitrary mixes of NB clones.

We have established a strategy for targeting specific NB lineages for clonal labeling (T. Awasaki, C.-F. Kao, Y.-J. Lee, C.-P. Yang and T.L., unpublished). The strategy is based on the GAL4-driven excision of a stop cassette within a pan-neuronal LexA driver in a subset of NBs, leading to a lineage-specific LexA driver in all subsequently derived progenies (Fig. 1A). Notably, a

[1]Howard Hughes Medical Institute, Janelia Farm Research Campus, 19700 Helix Drive, Ashburn, VA 20147, USA. [2]Neural Cell-Fate Determinants Section, NINDS, NIH, 35 Convent Drive, Bethesda, MD 20892, USA.
*These authors contributed equally to this work

‡Author for correspondence (leet@janelia.hhmi.org)

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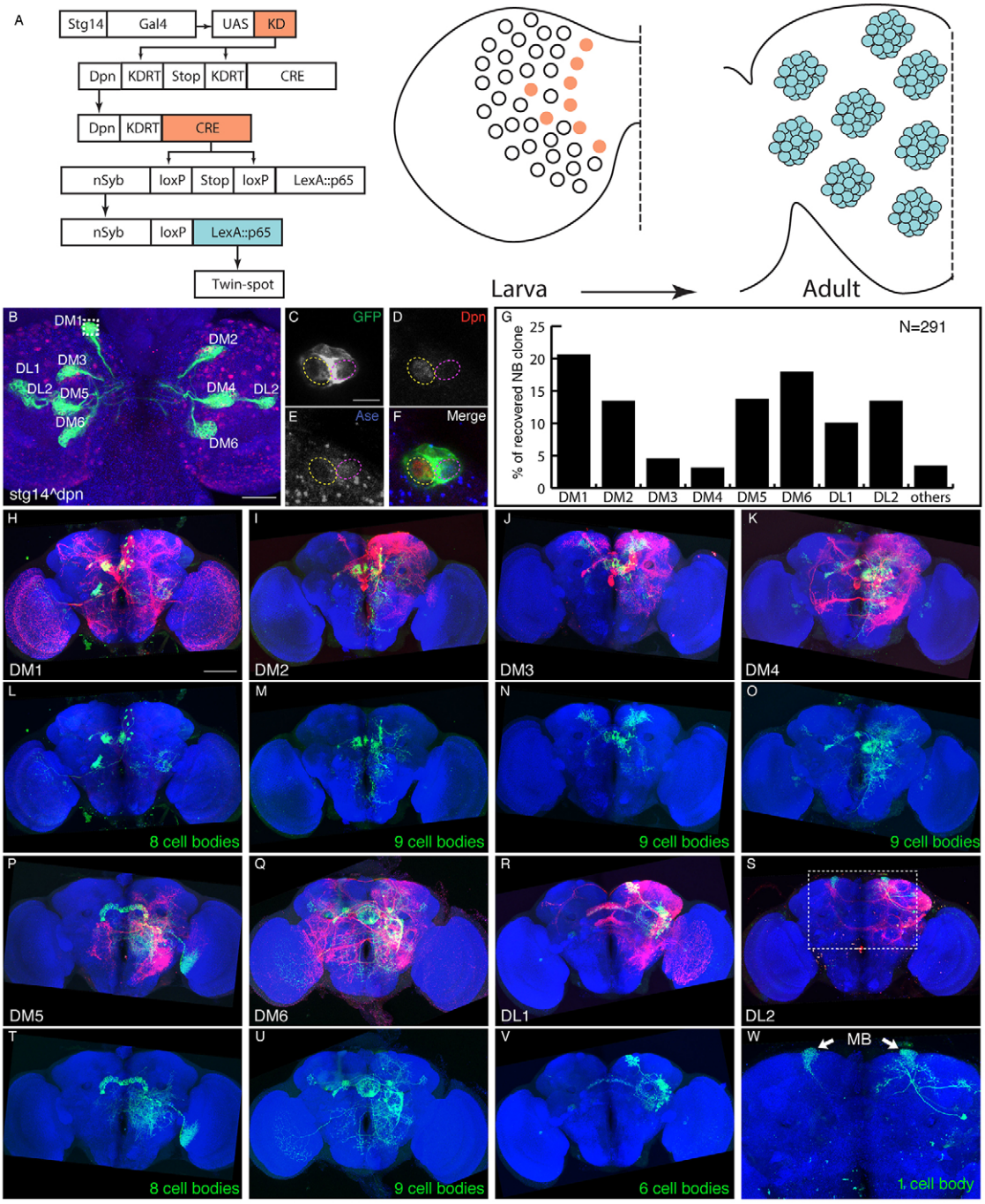

Fig. 1. Targeting *Drosophila* type II NBs for twin-spot clonal labeling. (A) Schematic illustration of the serial recombinase-dependent transgene activations via excision of the corresponding stop cassettes, enabling a pan-neuronal LexA::P65 driver specifically in the eight type II NB lineages (blue) due to stg14-triggered *dpn*-dependent Cre activity in the type II NBs (orange). (B-F) stg14-GAL4-dependent activation of dpn>stop>LexA::P65 allows selective labeling of type II NB lineages in larval brains. Note the GFP expression in various type II NB subsets across the brain lobes immunostained with anti-Dpn (red) and anti-Ase (blue). Magnified views show the Dpn-positive, Ase-negative DM1 NB (yellow dashed circle) in the left hemisphere. Note an Ase-positive INP (magenta dashed circle) lying adjacent to the DM1 NB. (G) The percentage of specific type II or all type I NB clones among the 291 NB clones recovered from 285 mosaic brains carrying the stg14^dpn lineage-restricted driver and experiencing clone induction at the first instar stage. (H-W) The twin-spot MARCM clones of type II lineages, induced shortly after larval hatching, consist of the first larval-born INP clone (green) paired with its parental NB clone (red) in nc82-counterstained adult brains (blue). The cell body numbers of the full-size INP clones are indicated. Note that the lone neuron in the DL2 INP clone selectively targets the tips of the paired mushroom body (MB) α lobes (W). W is an enlargement of the boxed area in S. Scale bars: 50 μm in B; 10 μm in C; 100 μm in H.

previously uncharacterized 975 bp genomic region (*stg-14*) located 8.5 kb upstream of the predicted transcription start site of *string* drives transient expression in embryonic type II NBs (data not shown). stg14-GAL4 filtered through a NB-specific *dpn* promoter (denoted stg14^dpn) can trigger serial recombination events specifically in type II NBs, leading to permanent activation of LexA drivers selectively in type II NB lineages (Fig. 1A-G). The strategy thus enables targeted clonal analysis in all postembryonic

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type II NB lineages without internal gaps, a feature that is hard to verify for standard GAL4 lines.

Twin-spot MARCM with the stg14^dpn lineage-restricted driver allowed us to recover clones selectively in the eight type II NB lineages (Fig. 1G). The DM1 to DM6 NB clones have cell body clusters near the midline with distributions spreading dorsoventrally on the posterior brain surface, whereas the DL1 and DL2 NB clones reside near the posterior dorsolateral corner (Yu et al., 2013). They exhibit complex morphologies but can be readily distinguished based on cell body distributions, neurite trajectories and neuronal elaboration patterns (Yang et al., 2013) (Fig. 1H-W). The complex yet stereotyped clone morphologies argue that each type II NB produces a characteristic set of diverse neuronal offspring in addition to glia (Izergina et al., 2009; Viktorin et al., 2011; Yu et al., 2013; Viktorin et al., 2013).

Identifying the first larval-born lineage-specific INP sublineages

To better determine the offspring diversity and describe how diverse neurons arise from serial INPs, we next examined the INP clones paired with the type II NB clones induced in newly hatched larvae and differentially labeled with twin-spot MARCM. This allowed us to identify the neuronal offspring of the first larval-born INP in each type II NB lineage. The full-size INP clones of DM1-6 and DL1 carry six to nine neuronal cell bodies (Fig. 1L-O,T-W), consistent with one INP budding off a short series of GMCs.

Interestingly, the DL2 NB clone is significantly smaller than other type II NB clones and its paired sister clone consistently carries only one viable neuron at the adult stage (Fig. 1W), reminiscent of some conventional type I lineages that exist as a lone hemilineage (Yu et al., 2010; Yu et al., 2013). Nonetheless, we could recover DL2 clones at comparable frequencies to the other type II NB clones with another type II NB-specific driver, giving us confidence in the identification of DL2 as the eighth type II NB lineage (data not shown).

The first larval-born INP (denoted INP1) clones exhibit lineage-characteristic stereotyped neurite trajectories (e.g. Fig. 2). These multicellular INP clones are complex and appear to carry diverse neuron types with distinct neurite trajectories. For instance, the DM1-4 INP1 clones acquire central complex (CX) as well as diverse non-CX elaborations (Fig. 1L-O). Furthermore, the INP1 clones of the super-exuberant DM1 and DM6 lineages exhibit most features of the parental NB clones despite drastic differences in clone size (fewer than ten neurons in INP clones versus more than 200 neurons in the paired NB clones) (Fig. 2). These phenomena argue that each INP contributes cells that include a significant fraction of the diverse cell classes observed in a given type II NB lineage.

However, the INP1 clones of most type II NB lineages exhibit domains of neurite elaboration that are unique to some offspring of the first larval-born INPs, as the following INP sublineages collectively labeled in the paired NB clones fail to innervate such INP1-specific domains. This is particularly obvious in the reduced DL2 lineage, where the lone mature neuron [probably one of the dopaminergic PPL1 neurons (Mao and Davis, 2009)] made by INP1 is the only larval-born DL2 neuron that innervates the tips of mushroom body (MB) α lobes bilaterally (Fig. 1S,W). We could readily detect INP1-unique elaborations in the complex DM1 and DM6 lineages as well. The INP1 clone of DM1 shows dense contralateral superior posterior slope (SPS) innervation (Fig. 2A-F), whereas the DM6 INP1 clone elaborates broadly alone in the ellipsoid body (EB) and contralateral optic lobe (OL) (Fig. 2G-L). These observations indicate that the INP1 clones are not only stereotyped but also unique, arguing that the ~40 serially derived INPs in a given type II lineage could be individually distinct.

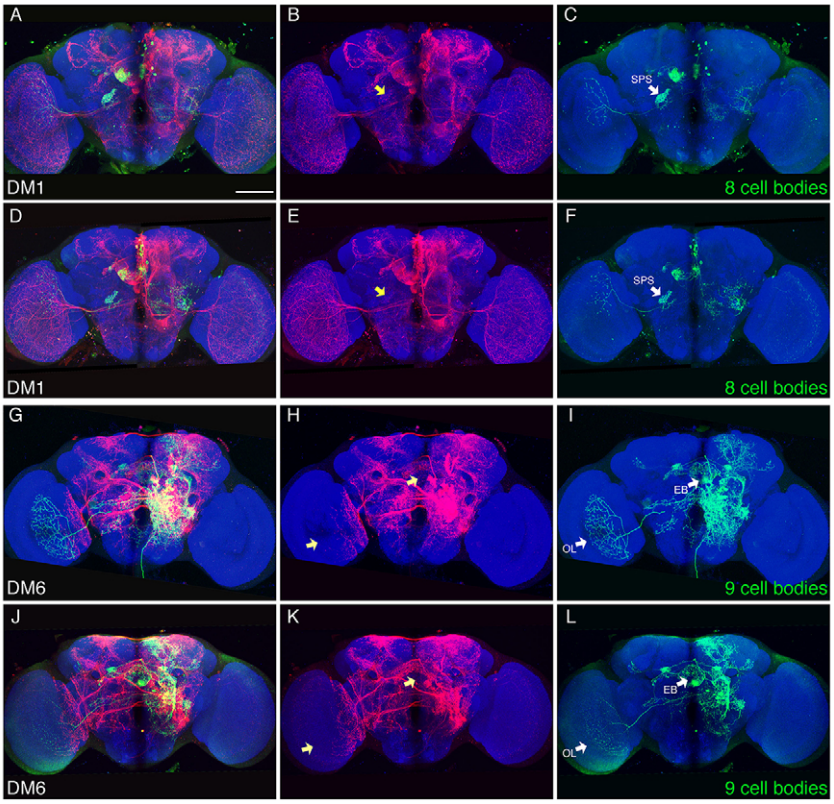

Fig. 2. Stereotypy and uniqueness of INP1 sublineages. (A-F) Two examples of DM1 twin-spot MARCM clones induced at early first instar carry indistinguishable full-size INP1 clones (green) paired with the remaining DM1 lineages (red). Note that the INP1-innervated SPS domain (white arrows) receives no innervation from the post-INP1 DM1 lineage (yellow arrows). (G-L) Two examples of DM6 twin-spot MARCM clones induced at early first instar carry indistinguishable full-size INP1 clones (green) paired with the remaining DM6 lineages (red). Note that the INP1-unique EB and broad OL elaborations (white arrows) are undetectable in the post-INP1 NB clones (yellow arrows). Adult brains were counterstained with nc82 monoclonal antibody (blue). SPS, superior posterior slope; EB, ellipsoid body; OL, optic lobe. Scale bar: 100 µm.

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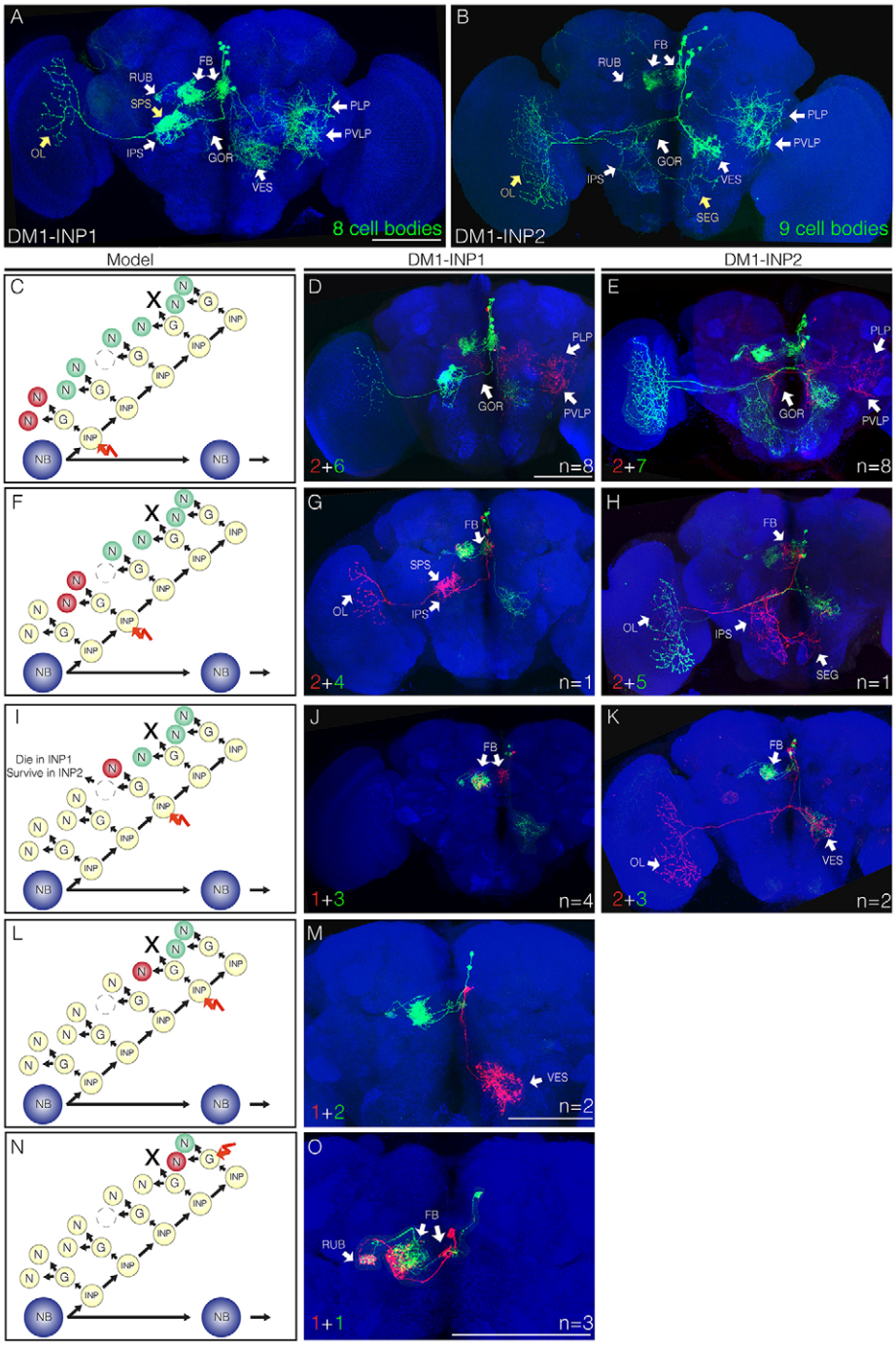

Fig. 3. Mapping neurons serially made by INP1 and INP2 of the DM1 lineage. (A,B) Full-size INP1 and INP2 clones of the DM1 lineage, accompanied by DM1 NB clones (not shown), exhibit common (white arrows) as well as unique (yellow arrows) domains of neurite elaborations. (C-O) Identification of INP subclones derived from DM1 INP1 or INP2 as judged from the presence of INP1- or INP2-characteristic neurite elaborations. The serially derived GMC clones (red) were paired with INP subclones of decreasing size (green), as schematized on the left. The offspring of GMC1 (red, D versus E), GMC4 (red in M) and GMC5 (O) are undistinguishable between the INP1 and INP2 sublineages, leaving the sublineage origin for the three last-born neurons (M,O) undetermined. By contrast, the GMC2 and GMC3 clones of INP1 versus INP2 (red, G versus H or J versus K) elaborate differentially due to fate differences in one of the two postmitotic neurons made by a given GMC (see Fig. 4). RUB, rubus; PLP, posterior lateral protocerebrum; PVLP, posterior ventrolateral protocerebrum; VES, vest; IPS, inferior posterior slope; GOR, gorget; SEG, subesophageal ganglion; FB, fan-shaped body. The numbers of samples are indicated. Scale bars: 100 μm.

Mapping sibling INP sublineages

To unravel the sibling INP diversity and reveal each neuron made by one INP, we further generated twin-spot MARCM clones in serial 4-hour windows from 8 hours to 48 hours after larval hatching (ALH). We selectively focused on the DM1 lineage for detailed sublineage analysis. The DM1 NB clones induced in first instar larvae prior to 16 hours ALH uniformly pair with the above identified INP1 clone (100%, *n*=59). Following clone induction between 16 and 24 hours ALH, we observe that all the DM1 NB clones that are unaccompanied by the unique INP1 clone instead pair with a different stereotyped INP clone, denoted as the INP2 clone (100%, *n*=9). The INP1 and INP2 clones share morphological features characteristic of the DM1 lineage (Fig. 3A,B). For example, they both innervate the CX, rubus (RUB), posterior lateral protocerebrum (PLP), posterior ventrolateral protocerebrum (PVLP), vest (VES) and the contralateral OL. However, compared with the INP1 clone, the INP2 clone carries one extra neuron and elaborates more ventrally in both the contralateral OL and central brain. Later derived INP clones remain analogous but distinguishable (our unpublished observations).

We proceeded to determine the similarities and differences in the offspring composition and sequence between the INP1 and INP2 sublineages. We collected twin-spot MARCM clones arising during INP self-renewing divisions. We could identify the paired subclones induced at the beginning of INP1 or INP2 proliferation based on the known sublineage-characteristic morphological features. We observe

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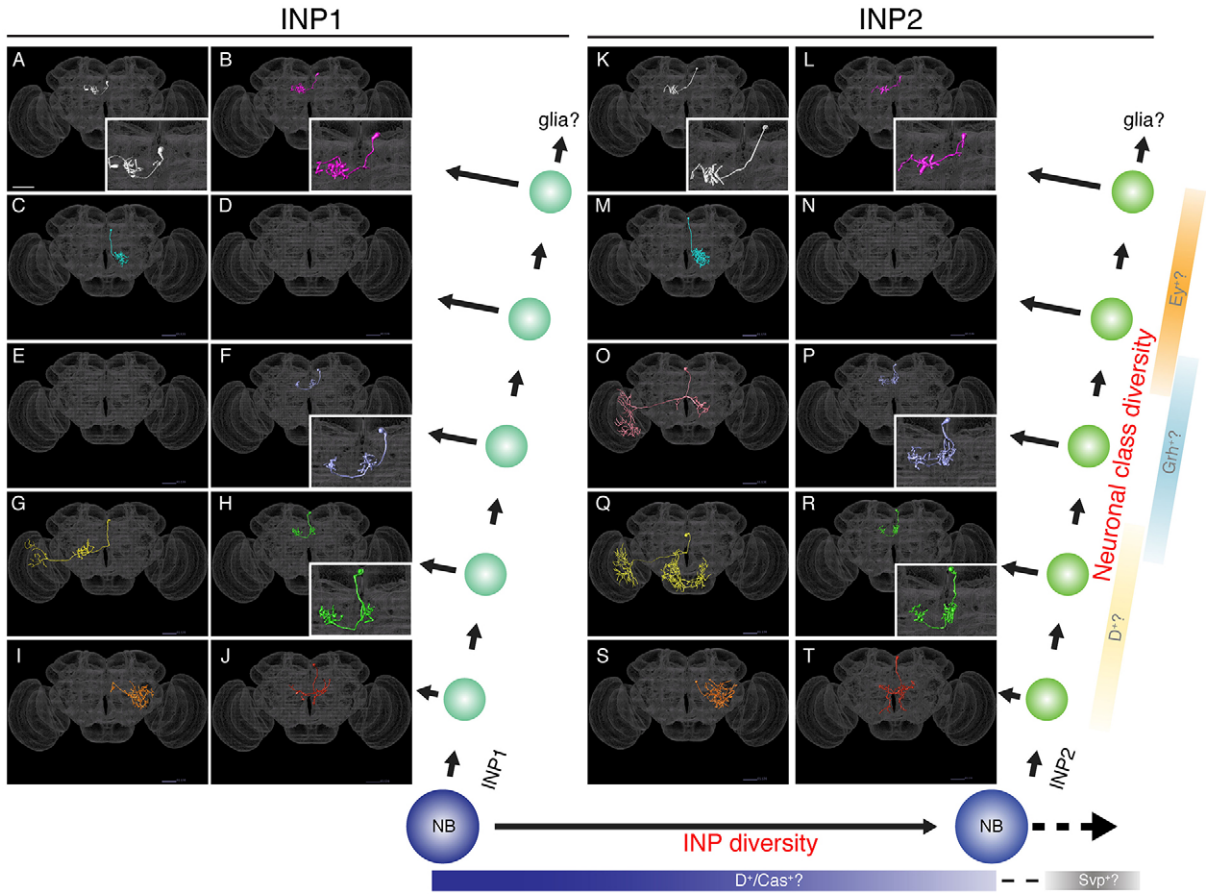

Fig. 4. Sibling INPs produce related invariant sequences of diverse neurons. Neurons made by the serial GMCs derived from the first two INPs of the larval DM1 lineage were individually traced and registered into a preselected adult fly brain template. Insets are magnified views of selected CX neurons. Known type II NB and INP temporal factors (Bayraktar and Doe, 2013) are shown. Scale bar: 100 µm; 20 µm in insets.

that a similar two-cell GMC clone may pair with a six-cell INP subclone that retains the INP1-unique SPS innervation (Fig. 3D) or a seven-cell INP subclone that shows INP2-characteristic broad OL and subesophageal ganglion (SEG) elaborations (Fig. 3E). The small GMC clones and their paired large INP subclones acquire distinct subpatterns of full INP clone morphologies, which clearly demonstrates both the diversity and invariant sequence in INP progeny. Close examination of the two-cell GMC clones and the paired single-cell clones derived from the first GMCs further reveals that the first GMC of both INP1 and INP2 consistently makes one strongly labeled neuron with lateral elaborations and one weakly marked neuron with medial projections (Fig. 3D,E and Fig. 4I,J,S,T).

We subsequently mapped the second GMC offspring for both the INP1 and INP2 sublineages (Fig. 3G,H). The GMC2 in both INP sublineages makes one OL neuron paired with a CX neuron (Fig. 4G,H,Q,R). But the OL neurons of INP1 and INP2 elaborate differently and underlie most of the sublineage distinctions. The INP1 OL neuron innervates the contralateral SPS and a dorsal domain within the contralateral OL (Fig. 4G), whereas the INP2 OL neuron arborizes broadly in the SEG and contralateral OL (Fig. 4Q). The third GMC offspring of INP1 and INP2 remain distinguishable and account for the difference in sublineage mature neuron numbers (Fig. 3J,K). GMC3 of INP1 makes only one viable CX neuron (Fig. 4F), whereas GMC3 of INP2 produces an analogous CX neuron as well as one extra OL neuron that targets VES and contralateral OL (Fig. 4O,P). The subsequently derived GMCs in both INP sublineages yield an undistinguishable set of three neurons, including one VES-targeting neuron plus two CX neurons (Fig. 3M,O and Fig. 4A-C,K-M). Therefore, mapping the sequence of individual neurons made by the DM1 INP1 and INP2 clearly shows that each INP sublineage consists of a sequence of very diverse neuron types that is repeated with slight modifications in the sequential INPs (Fig. 4). Not seeing the later-derived glia should not affect our neuron birthdating (Viktorin et al., 2011; Bayraktar and Doe, 2013) (our unpublished results).

Partial mapping of the DM6 INP1 and INP2 sublineages reveals similar phenomena. First, the INP1 and INP2 clones share morphological features characteristic of the DM6 lineage (supplementary material Fig. S1A,B). They elaborate analogously in superior medial protocerebrum (SMP), lateral accessory lobe (LAL), SPS, inferior posterior slope (IPS) and VES. But the INP1 clone solely targets the bulb/optic tubercle (BU/OTU) and contralateral OL, whereas the INP2 clone selectively innervates the fan-shaped body/superior clamp/superior intermediate protocerebrum (FB/SCL/SIP). In addition, they acquire distinct patterns of neurite elaborations in EB, gall and PLP. Second, the full-size nine-cell INP clones can be analogously partitioned into a two-cell GMC clone paired with a seven-cell INP subclone (supplementary material Fig. S1D,E). The GMC1 clones of DM6 INPs consistently carry one neuron extending dorsally into SMP or SMP/SIP and the other neuron projecting ventrally into VES. The following GMCs of the same INP sublineages, by contrast, make neurons innervating other neuropils.

These observations indicate once again that *Drosophila* type II NBs yield a series of analogous intermediate precursors that generate related sequences of diverse neurons.

Conclusions

We have learned that *Drosophila* type II NBs generate a series of INPs that in turn each produce an invariant sequence of neuronal classes. The same neuronal classes are produced in the same sequence repeatedly by the serial INPs, suggesting the reiterated use of related developmental fating programs in each INP sublineage. However, serial INPs only yield slightly different neurons based on the INP birth order, possibly governed by NB temporal factors (Bayraktar and Doe, 2013) and/or time-dependent environmental changes. It remains to be tested whether the *Drosophila* type II lineages are pre-fated along two dimensions – the sequence of INPs and the sequence of the progeny of each INP – to generate stereotyped arrays of diverse neurons.

MATERIALS AND METHODS

DNA cloning

The 975 bp *stg-14* genomic fragment, located 8.5 kb upstream of the *string* transcription start, was cloned using PCR primers: 5′ primer, GGTGTTCCAAATCGTTGGAC; 3′ primer, GGTATGAACATGTTCG-ATATCTACACC. The enhancer fragment was fused with a *Drosophila* synthetic core minimal promoter to make *stg14-GAL4*, as described (Pfeiffer et al., 2008).

Induction of MARCM clones

To obtain stage-specific twin-spot MARCM clones, we collected 0- to 4-hour-old larvae of *hs-FLP, dpn-KickdOUT-CrePEST; FRT[40A], lexAop-mCD8::GFP, lexAop-rCD2i/FRT[40A], lexAop-GFPi, lexAop-rCD2::RFP; stg14-GAL4/UAS-KD, nSyb-loxP-stop-loxP-LexA::P65*, which were cultured at 25°C and subsequently heat shocked at various times for 10-15 minutes at 37°C. Detailed information about the transgenes involved will be published elsewhere (T. Awasaki, C.-F. Kao, Y.-J. Lee, C.-P. Yang and T.L., unpublished).

Immunostaining, confocal imaging and data analysis

Brains dissected from 3- to 4-day-old adult flies were fixed and immunostained as described previously (Lee et al., 1999). Primary antibodies used include rabbit anti-GFP (1:1000; Invitrogen, A11122), mouse anti-GFP (1:50; Roche, 11814460001), rat anti-mCD8 (1:100; Invitrogen, MCD0800), rabbit anti-RFP (1:1000; Clontech, 632496), rabbit anti-Deadpan (1:50; gift of Chris Doe, University of Oregon, USA), guinea pig anti-Deadpan (1:1000; gift of Fengwei Yu, originally from Jim Skeath, Washington University, USA), guinea pig anti-Asense (1:100; gift of Jurgen Knoblich, IMBA, Austria) and mouse anti-Bruchpilot (nc82 monoclonal antibody; 1:50; Developmental Studies Hybridoma Bank). Fluorescent signals of whole-mount larval or adult brains were collected by Zeiss LSM 710 confocal microscope and processed with Adobe Photoshop to optimize image quality. Isolated single neurons were traced semi-automatically by neuTube (Kim et al., 2011; Zhao et al., 2011). The traced neurons in Fig. 4 were registered into a preselected adult fly brain template using BrainAligner with Vaa3D (Peng et al., 2011).

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Competing interests

The authors declare no competing financial interests.

Author contributions

Y.-C.W. carried out experiments and data analysis. J.S.Y. performed pilot studies. R.J. optimized fly brain immunostaining and confocal imaging. Q.R. assisted data analysis. Y.-J.L. assisted data acquisition. H.L. assisted fly genetics. T.B. and W.F.O. identified stg-14. T.L. conceived the study, designed and coordinated experiments and wrote the manuscript.

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Supplementary material

Supplementary material available online at
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Figure S1. Partial mapping of the DM6 INP1 and INP2 sublineages

(A,B) Full-size INP1 and INP2 clones of DM6 lineage, accompanied by DM6 NB clones, not shown, exhibit common (white arrows) as well as unique (yellow arrows) domains of neurite elaborations. Note their acquisition of distinct elaborations in gall, EB, and PLP (magenta arrows).

(C-E) Mitotic recombination at the beginning of INP proliferation, as schematized in [C], allows differential labeling of the first GMC's offspring (red) versus the remaining INP sublineage (green). The DM6 INP1 or INP2 subclones were identified based on the demonstrated morphological features characteristic of the full-size DM6 INP1 and INP2 clones (yellow and magenta arrows in [A] and [B]). Note the GMC1 clones of sibling INPs look alike but distinct from their paired INP subclones. Primary elaborations of the GMC1 clones are indicated. Scale bar: 100 μm.