Clonally Related Interneurons Are Not Constrained by Functional or Anatomical Boundaries

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Introduction

In 2015, two papers were published in *Neuron* (Harwell et al., 2015, and Mayer et al., 2015) that jointly argued that interneuron lineages were dispersed across functional and structural boundaries. These conclusions were challenged by the laboratory of Songhai Shi (Sultan et al., 2016), and this Short Course chapter presents our response. In it, we discuss ongoing single-cell approaches that combine whole-genome analysis and lineage to take the next step toward understanding the possible links among interneuron lineage, cell type, and position within the brain.

During development, excitatory principal neurons and inhibitory interneurons assemble within the mammalian cortex and integrate into common circuits. However, a fundamental question in developmental neuroscience remains whether clonally related interneurons, like excitatory neurons, maintain a coherent relationship with their siblings while populating specific cortical areas and the local columnar architecture therein. Our laboratory and a copublished article (Harwell et al., 2015; Mayer et al., 2015) independently took advantage of a lineage fate mapping method devised by the Cepko Lab. With this method, a replication-defective retroviral library that contains a highly diverse set of DNA barcodes can be used to tag dividing progenitor cells during embryonic development, thereby permitting the unambiguous determination of lineage relationships across individual cells in the adult. Both studies reported that interneurons derived from a single progenitor lineage within the forebrain disperse widely across both functional and anatomical structures. As outlined in their upcoming article in *Neuron*, the laboratory of Dr. Shi (Sultan et al., 2016) further analyzed our datasets and concluded that clonally related interneurons are not "randomly dispersed," and we agree with this conclusion. In fact, we never claimed that interneuron clones "randomly disperse" either within or across brain structures. Rather, we reported a finding consistent with Sultan et al. (2016) that ~30% of clones spanned more than one brain structure, providing clear cases in which progenitor lineage is not predictive of an interneuron's ultimate anatomical or functional fate. In addition, we found that the spatial distribution of clones is similar among progenitors regardless of whether they share a lineal relationship. Based on our findings, we conclude that the integration of interneurons into functional cortical areas is unlikely to be constrained by lineage.

The mammalian cortex is subdivided into areas devoted to vision, sensation, audition, and other functions. Each area can be further divided physiologically into smaller units or functional columns. Excitatory and inhibitory neurons (the two main cell types of the cortex and hippocampus) have very distinct embryonic origins (Anderson et al., 1997) and have segregated into separate lineages by the time the primary prosencephalon has developed into the secondary prosencephalon (Rubenstein et al., 1998). Excitatory cells are derived from the dorsal telencephalon or pallium. Consecutive rounds of asymmetric cell division produce lineage-related sister excitatory neurons that migrate short distances toward the pia and into the overlaying developing cortical plate. After migration, spatially organized vertical clusters of excitatory sibling neurons (referred to as "clonal units") form functional columnar microcircuits in the neocortex (Noctor et al., 2001; Li et al., 2012). In contrast, inhibitory cells derive entirely from the ventral telencephalon or subpallium (Marin and Rubenstein, 2001; Fishell and Rudy, 2011), most prominently from the medial and caudal ganglionic eminences (MGE and CGE, respectively), and migrate over large distances to integrate into the developing cortex, hippocampus, or other subcortical forebrain structures.

Conflicting Results from Four Recent Studies Examining Interneuron Lineages

Despite the technical difficulties associated with fate mapping interneuron lineages resulting from their complex migration patterns, four recent studies (Brown et al., 2011; Ciceri et al., 2013; Harwell et al., 2015; Mayer et al., 2015) have endeavored to explore whether clonally related interneurons are selectively positioned within cortical units, similar to what is observed in excitatory neurons. If clonally related interneurons were confined to discrete anatomical brain units (e.g., columns of the cortex), this would support the idea that cell lineage is dictating the integration of interneurons into functional cortical networks.

All four groups agreed that before migration, the majority of interneurons are generated from symmetric and asymmetric divisions of MGE progenitor cells, leading to radially aligned interneuron precursors being symmetrically aligned in proximity to each other (Brown et al., 2011; Ciceri et al., 2013; Harwell et al., 2015; Mayer et al., 2015). Postmitotic interneurons

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reach their final positions within the cortex through long-range tangential migration that requires them to travel 100 times farther than excitatory pyramidal neurons to reach the cortical plate.

However, the four studies drew different conclusions about how lineage contributes to the final location of interneurons after long-range migration. Brown et al. (2011) and Ciceri et al. (2013) described clonal clusters in the cortex that were sufficiently compact to suggest that they were confined by functional boundaries. Specifically, Brown et al. suggested that presumptive clones were aligned into functional columns, very similar to their excitatory counterparts (Brown et al., 2011; Yu et al., 2012), raising the possibility of a lineage-dependent functional matching in the organization of inhibitory and excitatory neurons (Brown et al., 2011). Ciceri et al. (2013) did not detect such radial clusters but rather described exclusively laminar clusters. In contrast, Mayer et al. (2015) and Harwell et al. (2015) both concluded that clonally related interneurons can disperse across anatomical and functional boundaries within the forebrain and are not restricted to narrow cortical columns or lamina. Notably, Mayer et al. (2015) and Harwell et al. (2015) agreed that sibling interneurons reside in a volume that far exceeds functional cortical units, such as the whisker barrels (Bruno et al., 2003) of the somatosensory cortex (the average distance between pairs of sibling neurons was >2 mm in Mayer et al., 2015). These data imply that the integration of interneurons into functional units is unlikely to be determined by lineage.

Can Cluster Analysis Be Used to Determine Lineal Relationships Between Interneurons?

A common feature of all studies considered above is that interneuron progenitors in the MGE of mouse embryos were labeled through infection using very similar fluorescently tagged retroviruses. What then explains the disparate results reported in these four investigations? Discrepancies almost certainly arose from the different methods used to assess and define interneuron clonality. Mayer et al. (2015) and Harwell et al. (2015) used a replication-defective retroviral library containing a highly diverse set of DNA barcodes, an approach pioneered by Walsh and Cepko in the early 1990s (Walsh and Cepko, 1993), to determine lineal relations between labeled interneurons. Recovering the barcodes from the mature progeny of infected progenitor cells enabled Mayer et al. and Harwell et al. to unambiguously determine the lineal relationship between clones regardless of their geometric distribution within the brain. In contrast, Brown et al. (2011) and Ciceri et al. (2013) used a combination of approaches, including (1) time-lapse imaging (before migration), (2) mixing of red and green retroviruses, and (3) presumptive clonal labeling with low-titer retrovirus injections followed by the use of geometric criteria to infer lineal relationships among retrovirally labeled neurons. For the following reasons, we believe that none of the aforementioned methods used by Brown et al. (2011) and Ciceri et al. (2013) reliably indicated lineal relationships among interneurons.

First, whereas in principle time-lapse imaging could be used to determine lineal relationships, this approach is impractical, given both the distances involved and the protracted time over which interneurons migrate from their birth to their settling position. Second, the use of red and green retroviruses is confounded by technical difficulties that, when addressed by Ciceri et al. (2013), revealed that assigned clusters of interneurons are polyclonal in nature. In brief, they reported that when retroviruses encoding green fluorescent protein (GFP) and mCherry were mixed before ultracentrifugation, "most clusters were likely to include cells from a different progenitor (i.e., a different fluorescent protein), even at very limiting dilutions." The authors concluded that "this strongly suggested that lineage relationships are not exclusive determinants of interneuron clustering." Third, whereas low-titer retroviral injections can in principle be used to determine lineal relationships, in practice this proves untenable. If one could reliably label a single progenitor with a single injection, it would of course be possible to trace interneurons in the forebrain, even if individual siblings pursued drastically different migration paths. However, the labeling of a single progenitor cell cannot be guaranteed using current technology. Retroviral labeling of multiple progenitor cells unavoidably results in both lumping errors (clustered cells that are not clonal) and splitting errors (dispersed cells that are clonal but are not recognized as such), particularly if cells undergo complex migration.

Brown et al. (2011) attempted to minimize lumping and splitting errors by using low-titer retroviral injections "to label dividing progenitor cells in the ventricular zone…at clonal density." Given this claim, we were surprised when we looked at the raw data provided by Dr. Shi (Figs. 1*A*, *B*) to see that individual brains showed >500 labeled cells—far exceeding what our analysis indicated would allow for "clonal labeling." These data, we believe, preclude the assignment of lineage using the geometric criteria used by Brown et al. (2011) and Ciceri et al. (2013). More specifically, to assign lineage after interneuron

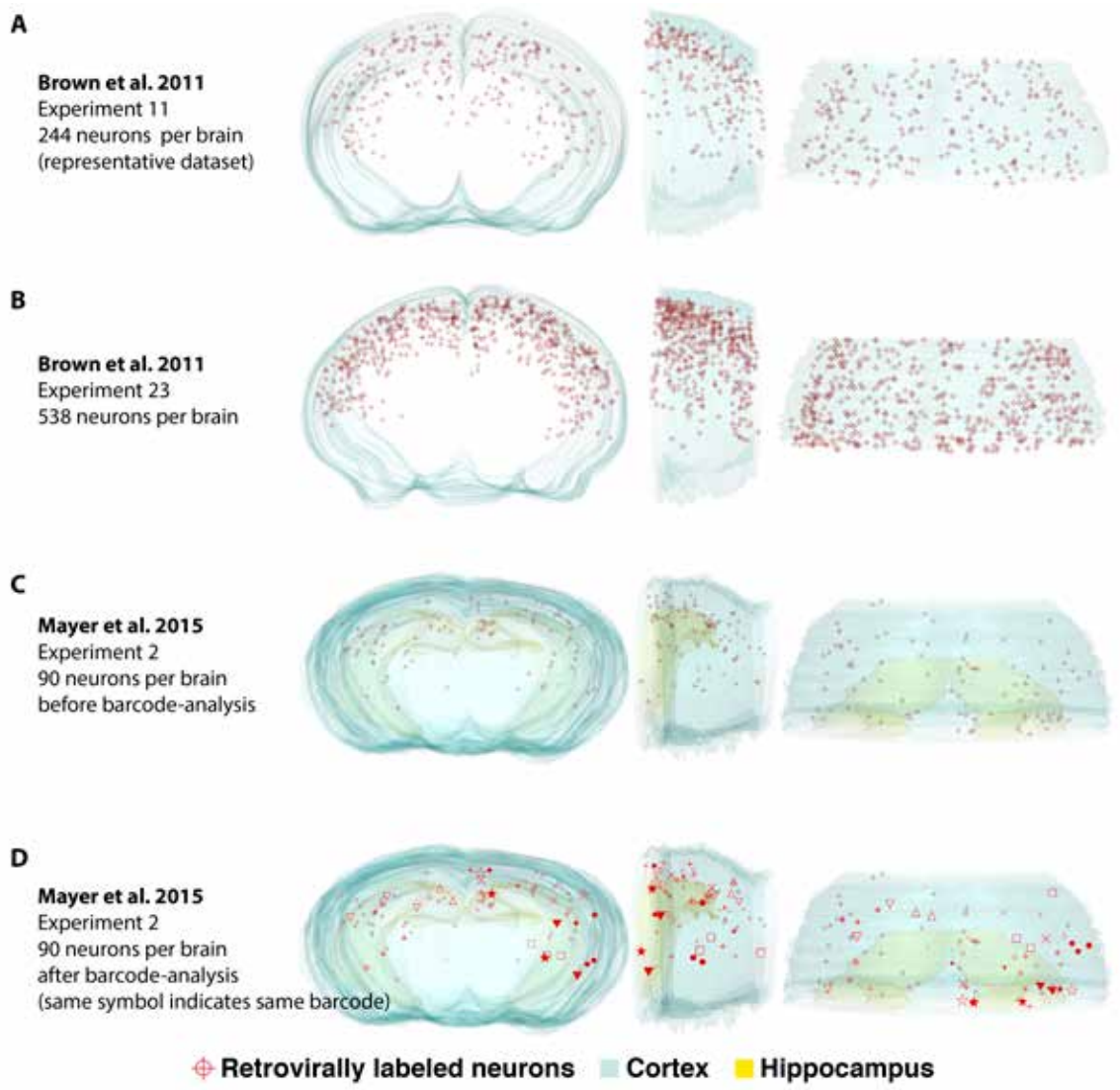

Figure 1. Comparison of the distributions of retrovirally infected interneurons in Mayer et al. (2015) and Brown et al. (2011). ***A, B,*** Two experimental datasets from Brown et al. are shown. Three-dimensional reconstructions of the distribution of cortical interneurons in a postnatal *Nkx2.1*$^{Cre/+}$;*R26*$^{LSL\text{-}TVAiLacZ/+}$ mouse infected with retroviruses expressing enhanced green fluorescence protein (EGFP). Datasets in Brown et al. contained ≤538 data points per brain. To predict clonal relations of inhibitory interneurons, Brown et al. applied spatial parameters based on the observed distributions of excitatory neuron clusters (not shown). ***C, D,*** Three-dimensional reconstructions of a representative dataset reproduced from Mayer et al. (2015), illustrating the distribution of cortical interneurons in a postnatal *Nkx2.1*$^{Cre/+}$;*R26*$^{LSL\text{-}TVAiLacZ/+}$ mouse that was infected with a retroviral library. The same dataset is shown, before ***C*** and after ***D*** determination of clonal relations based on retroviral barcodes. The dark red symbols (stars, circles, or triangles) represent single-cell clones (i.e., neurons harboring a barcode that occurred only once in the dataset); light red symbols represent multicell clones, whereby symbols with the same shape indicate the location of sister interneurons (i.e., neurons with the same barcode).

labeling and migration, both Brown et al. and Ciceri et al. compared the distance from each interneuron to its closest neighbor (nearest neighbor distance [NND]) with a randomly computer-simulated dataset to test whether the labeled interneurons were clustered. Ciceri et al. then calculated the number of clusters in the experiment using a threshold distance value that maximized the difference between the number of clusters observed in the experimental dataset and the mean number of clusters in 100 simulated populations of randomly distributed neurons. Brown et al. used spatial parameters that picked up excitatory neuron clusters to predict clonally related inhibitory interneuron clusters.

Because these methods require that any "clonal"

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group of cells be constrained to a specified geometric area, as a matter of principle, these methods cannot be used to study dispersed clones that reside in different forebrain structures or distant locations within the neocortex. In addition to missing clonal dispersion across areas, our findings (as well as those of Harwell et al., 2015) demonstrated that the use of such geometric criteria also failed to predict clonality of interneurons within the cortex. When local clusters are deemed to be clonal clusters, lumping errors are a major confounding factor, particularly for datasets with a large number of total neurons (e.g., those used in Brown et al., 2011; Figs. 1*A, B*). This is because as the number of labeled neurons in a dataset increases, the chance that a nonclonally related cell will be found nearby clonally related cells also increases. Sultan et al. (2016) recognized this point, as they stated, "a clone forming a local cluster does not preclude the presence of nearby non-clonally related interneurons.... The more data points, the shorter the distance in general between them. Therefore, it is crucial to take into consideration the total number of data points in each dataset." Even with much lower rates of infections per brain (Fig. 1C), Mayer et al. (2015) and Harwell et al. (2015) reported a large number of interneurons that were nearest neighbors but not clonally related (i.e., they had different DNA barcodes, indicating that they originated from different progenitors) (see dendrogram analysis in Mayer et al., 2015). In their recently published article in *Neuron*, Harwell et al. provided an additional detailed analysis, showing that the spatial parameters used in Brown et al. (2011) to cluster interneurons had failed to identify lineal boundaries in either our dataset or their own.

Complications Arising from the Analysis by Sultan et al.

As outlined in their upcoming article in *Neuron*, the lab of Dr. Shi (Sultan et al., 2016) further analyzed our datasets and concluded that clonally related interneurons in our datasets were not randomly dispersed. Their study implied that this contradicted our findings, attributing to us conclusions to which we do not subscribe. We hold that the real discrepancy between our conclusions and those of Sultan et al. is semantic, coming down to how we precisely define a cluster. "Clusters," per definition, are a group of things that occur close together. In the cases of Brown et al. (2011) and Sultan et al. (2016), clusters were determined geometrically, as groups of cells that occur closer to each other than predicted in a random distribution (random computer-simulated cells). We completely agree that retrovirally labeled cohorts of interneurons appear clustered when compared with a randomly distributed (computer-simulated) group of data points, but given the biological constraints placed on interneuron development, this should come as no surprise. For example, it is known that interneurons' ultimate location in the brain is heavily influenced by several factors: (1) their position and time of birth (Miyoshi et al., 2007), (2) prescribed paths of migration (Tanaka et al., 2006; Marin, 2013), and (3) stereotyped radial migration from the marginal and subventricular zones to the cortical plate (Miyoshi and Fishell, 2011). All these factors indicate that although the dispersion of interneurons is perhaps stochastic, it is also tightly regulated, and therefore a random dispersion model will be grossly inaccurate.

Are Interneuron Clones Preferentially Clustered?

Similar to the analysis done in Mayer et al. (2015), but for cortical clones only, we further examined whether the average distance between pairs of lineage-related interneurons is preferentially reduced compared with unrelated interneurons. We found that the results for average distance between pairs of neurons is not influenced by the total number of data points in individual datasets (unlike, e.g., the NND; Fig. 2*A*), thus providing a robust measure for comparing clonally related and unrelated cells. Notably, both lineage-related and lineage-unrelated interneurons were labeled at the same time and with the same method, ensuring that they shared similar birthdates and migratory trajectories. The "intraclonal distance" was calculated as the average distance between pairs of clonally related interneurons, and the "interclonal distance" was calculated as the average distance between pairs of unrelated cells within one hemisphere (Fig. 2*B*). "Pairs of unrelated cells" included the distance between all possible pairs of interneurons with different barcodes: (1) individual members of "multicell clones" with different barcodes, (2) "single-cell clones," and (3) individual members of "multicell clones" and "single-cell clones." Significantly, the average distance between 40 pairs of clonally related interneurons in the cortex of P16 mice (average distance [AD] = 2134 ± 213, SEM) was not statistically different from 926 pairs of clonally unrelated interneurons (AD = 2145 ± 34, SEM; $p > 0.9$, Kruskal–Wallis test, multiple comparison; $p = 0.6$, Mann–Whitney nonparametric t test) (Fig. 2C). When we broke down the analysis by dataset (i.e., for each retrovirally infected brain), we did not detect a statistical difference despite the low numbers of clonally related pairs in each analysis ($p > 0.1$ in all three datasets, Mann–Whitney nonparametric t test) (Fig. 2*D*). Taken together, our results indicate

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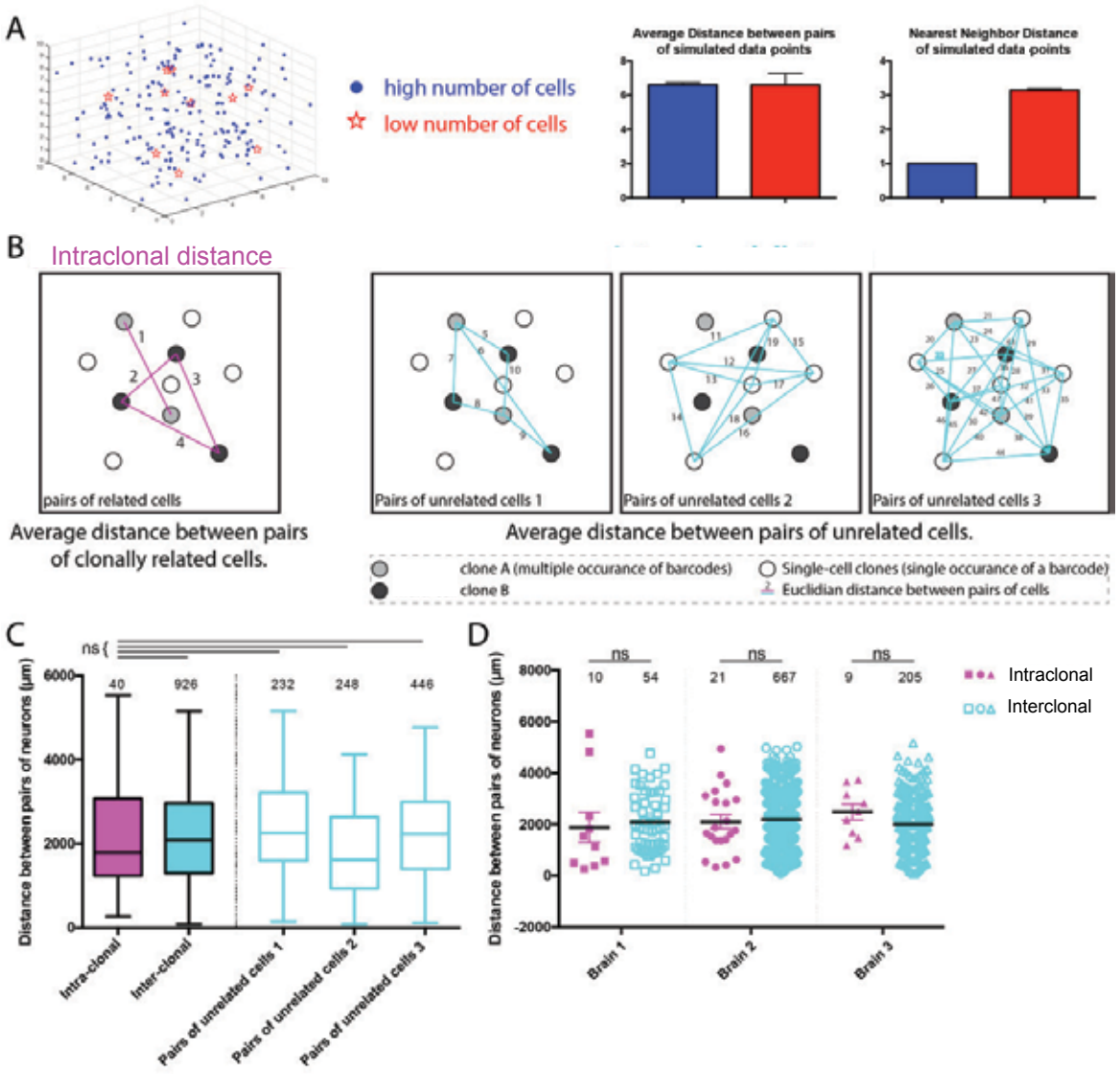

Figure 2. Interneuron clones within the cortex in Mayer et al. (2015) are not spatially segregated when compared with a biologically appropriate control group. ***A,*** The NND decreases as the number of cells per dataset increases. Notably, the AD between pairs of neurons is not influenced by the total number of data points in individual datasets. To illustrate this principle, NNDs and ADs were calculated for simulated datasets containing a high number of cells (200; blue dots) and a low number of cells (10; red five-pointed stars) in a given volume; $N = 100$ simulations; ***B,*** Schematic illustration showing an analysis similar to that done in Mayer et al. (2015), except that in the present case, only included cortical intraclonal and interclonal distances were calculated for interneurons. The intraclonal distance was calculated as the average distance between pairs of clonally related interneurons. The interclonal distance represents the sum of distances between (1) individual members of "multicell clones" with different barcodes, (2) "single-cell clones," and (3) individual members of "multicell clones" and "single-cell clones." ***C,*** Box-and-whiskers plot of the intraclonal and interclonal distance. Whiskers indicate minimum-to-maximum values. All three datasets from Mayer et al. were included in this analysis. The interclonal distance represents the sum of the three pairwise comparisons between (1) multicellular but unrelated clones, (2) single-cell clones, and (3) individual members of multicellular and single-cell clones (***B***). No significant difference in separation was observed when comparing intraclonal and interclonal distances (Kruskal–Wallis test, multiple comparison; Mann–Whitney nonparametric *t* test). The number above the boxes indicates the number (*n*) of interneuron pairs. ***D,*** Scatter plot of intraclonal and interclonal distances by brain (1–3). No significant difference in separation was observed when intraclonal and interclonal distances were compared (Kruskal–Wallis test, multiple comparison; Mann–Whitney nonparametric *t* test); the number above the boxes indicates the number (*n*) of interneuron pairs.

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that in general, clonally related cells are not located closer to each other than a biologically similar group of nonrelated interneurons.

Dendrogram Analysis Has Limited Utility in Determining Clonal Clusters

As mentioned above, lumping errors and splitting errors cannot be avoided if clonal clusters are defined geometrically because these methods implicitly assume that neighboring cells are clonally related. The separation between cells that are clustered versus not clustered is strongly influenced by the total number of data points in the dendrogram. In Mayer et al. (2015), we performed a dendrogram analysis to illustrate that this is an inherent problem when using geometrical methods. In brief, we grouped GFP-labeled neurons, regardless of lineage, by their proximity and displayed the results in dendrograms (Mayer et al., 2015). We then labeled the neurons according to their lineage relationship (i.e., barcode identity). Despite the fact that we labeled a relatively small number of neurons (e.g., much lower than in Brown et al., 2011; Fig. 2) in our dataset, only 52% of clones (12 out of 23) formed closest nearest neighbors (lowest hierarchical branch in the dendrogram; Mayer et al., 2015). In addition, a number of the clones that were closest nearest neighbors on the dendrogram had at least one "split" sibling on a far branch on the dendrogram. Sultan et al. (2016) reached a very similar result: they also found that 52% of clones (14/27) were closest nearest neighbors. It is critical to note that our results preclude the use of dendrogram analysis to determine the lineage relationships between neurons, and it was never our intention to use it for that purpose.

We would like to address the "error corrections" made by Sultan et al. (2016) when they reanalyzed the dendrogram analysis presented in Mayer et al. (2015). In particular, Sultan et al. stated that we failed to add clone #32 to our dendrogram. However, calling this an "error correction" is inaccurate because we deliberately excluded this clone from our analysis. Clone #32 was located within the olfactory bulb, and the dendrogram analysis in Mayer et al. "focused on cortical, hippocampal, and striatal clones only." In another such "error correction," Sultan et al. noted that clone #12 contained three cells in the cortex and three cells in the hippocampus, requiring them to "add all six clones to the dendrogram." This statement implies that we incorrectly excluded all these cells from our dendrogram, which again is inaccurate. We deliberately divided clones that crossed anatomical boundaries for analysis within brain structures.

The Use of Euclidian Distance Measurements

Sultan et al. (2016) noted that the use of Euclidian distances in structures such as the cortex is problematic, as it is clear that in many if not most cases, migration along straight lines (e.g., in cases where such trajectories would cross ventricles or sulci) is not biologically tenable. Nonetheless, all distances between pairs of neurons described in Mayer et al. (2015) as well as Sultan et al. (2016) and Brown et al. (2011) were calculated as Euclidian distances. Given the impossibility of determining more realistic trajectories, this approach is at least systematic, and by its nature chronically underestimates the real distances between neurons. This only strengthens our conclusion that clonal dispersion does not respect functional boundaries, as properly corrected measurements of the distance between clones would only be larger rather than smaller.

Interneuron Clones Can Span Multiple Brain Structures

Although the results of Mayer et al. (2015) and Harwell et al. (2015) demonstrated that interneuron clones are not obliged to populate particular anatomical structures, this does not rule out the possibility that they are predetermined to occupy particular brain regions. Sultan et al. (2016) discussed this point as follows:

1. Should lineage relationship have no influence on interneuron distribution, the relatively total interneuron output to different forebrain structures and the small clone size dictate that virtually all clones must be located in the cortex, the cortex and hippocampus, or the cortex and striatum. Interestingly, a significant fraction of clones was observed to be restricted to the hippocampus or striatum (Mayer et al., 2015), suggesting that some MGE/PoA [medial ganglionic eminence/preoptic area] progenitors specifically produce interneurons destined for these two brain structures.

2. While it is evident that the majority (~66% in Mayer et al. and 80% in Harwell et al.) of clones are located within one brain structure, i.e. the cortex, some are dispersed in more than one brain structure. However, this clonal dispersion largely occurs between the cortex and hippocampus, the two highly related forebrain structures emerging

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side-by-side in the dorsal telencephalon. The same tangential migration routes are responsible for interneuron distribution in the cortex and hippocampus (Ayala et al., 2007; Marin and Rubenstein, 2001, 2003). In comparison, only a small fraction (~12.5% in Mayer et al. and 20% in Harwell et al.) of clones is dispersed between developmentally unrelated brain structures such as the cortex and striatum, or globus pallidus, or olfactory bulb.

Although these statements are factual, understanding their implications requires a more nuanced analysis. Both the absolute size of the cortex, hippocampus, and striatum as well as the density of interneurons within these structures differ dramatically. For example, 20% of the cells within the cortex and hippocampus are interneurons (Fishell and Rudy, 2011), whereas the percentage of interneurons within the striatum is only 3% (Marin et al., 2000; Tepper et al., 2010). These facts demonstrate that even if interneurons were randomly distributed to different structures, probabilistically, they would be preferentially found in the cortex. That said, we reiterate that we do not believe that the distribution of interneurons is random. But what rules then underlie the distribution of discrete interneuron lineages? Our results definitively indicate that if interneuron lineages do have a covert logic as to how they populate different structures, clearly the rules of allocation are not as simple as an interneuron lineage being earmarked for cortex or hippocampus per se. Further examination of interneuron lineages will be required to address whether there is a degree of predetermination in the positioning of sibling neurons derived from a common lineage.

Final Remarks

We have shown here and in previous work (Mayer et al., 2015) that clonally related interneurons are no more closely clustered than nonlineally related interneurons (proximally generated brethren). These findings, of course, neither should nor do end the debate as to whether lineage contributes to the development, subtype differentiation, or connectivity of interneurons. Our results were limited by the fact that the lineages we assembled were only partially reconstructed, so we can say nothing regarding the fate of those sibling cells that we failed to recover. In addition, we know startling little about the phenotypic identity and nothing about the connectivity of clonally related siblings, both of which would be fascinating to explore. We would, however, implore any further examination of lineage to confine itself to methods that provide a high degree of confidence about the lineage relationships of cells designated as clones.

Acknowledgments

We are grateful to Xavier Jaglin, Robert Machold, and Timothy Petros for comments on this manuscript. Research in the Fishell Laboratory is supported by the National Institutes of Health (grants NS081297, MH095147, and P01NS074972) and the Simons Foundation.

This paper was excerpted and modified with permission from a previously published article: Mayer, C; Bandler, RC; Fishell, G (2016) Lineage is a poor predictor of interneuron positioning within the forebrain. Neuron 92:45 – 51, copyright 2016, Elsevier.

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