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UNIVERSITY OF CALIFORNIA SAN DIEGO

Distinct developmental trajectories and molecular signatures of layer II excitatory neurons in
murine entorhinal cortex

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Alanna Elizabeth Koehler

Committee in charge:

Professor Robert F. Hevner, Chair
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2022

The dissertation of Alanna Elizabeth Koehler is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2022

DEDICATION

To K.G., M.S., and J.S. You know why.

*To my loving family for their everlasting support, late-night cross-country phone calls,
and for raising me to be my most stubborn, confident, and genuine self.*

*To the incredible, formidable women going through this journey alongside me,
for the Friday night beers, ocean excursions, and the will to keep going.
T.L., G.G., C.A., M.B., and R.L*

*To R.N.J. for seeing my full potential, for teaching me what good
science looks like, and for never treating me like a “woman” in the field.*

*To my boy Dart for the laughs, the love, the goofy noises, and a thousand loops around
Fiesta Island. I promise to buy you a doggy raincoat for our new life on the East Coast.*

*And to my dear Ida Bean for being uniquely, unapologetically you and showing me the meaning
of unconditional love. We found each other at just the right time, and I lost you far too soon. I
miss you every day. See you over that Rainbow Bridge, baby girl.*

EPIGRAPH

“To be gritty is to keep putting one foot in front of the other. To be gritty is to hold fast to an interesting and purposeful goal. To be gritty is to invest, day after week after year, in challenging practice. To be gritty is to fall down seven times and rise eight.”

— Angela Duckworth

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LIST OF ABBREVIATIONS

EC	Entorhinal cortex
HPF	Hippocampal formation
AD	Alzheimer's disease
L	Layer
SN	Stellate neuron
PN	Pyramidal neuron
NEC	Neuroepithelial cell
RGC	Radial glial cell
IPC	Intermediate progenitor cell
RNA	Ribonucleic acid
snRNAseq	Single nucleus RNA sequencing
DG	Dentate gyrus
HP	Hippocampus proper
SUB	Subiculum
PrS	Presubiculum
PaS	Parasubiculum
MTL	Medial temporal lobe
MEC	Medial entorhinal cortex
LEC	Lateral entorhinal cortex
CA	Cornu ammonis
E	Embryonic day (day post-fertilization)
SVZ	Sub-ventricular zone

TLE	Temporal lobe epilepsy
NFT	Neurofibrillary tangle
β A	Beta amyloid
IHC	Immunohistochemistry
ISH	<i>In situ</i> hybridization
FISH	Fluorescent <i>in situ</i> hybridization
smFISH	Single molecule fluorescent <i>in situ</i> hybridization
LD	<i>Lamina dissecans</i> (EC layer 4)
ATACseq	Assay for transposase-accessible chromatin with sequencing
BrdU	5-bromo-2'-deoxyuridine
FACS	Fluorescence-activated cells sorting
UMAP	Uniform manifold approximation and projection
PCA	Principal component analysis
PC	Principal component
Ex	Excitatory neurons
In	Inhibitory neurons
NNC	Non-neuronal cells
OPC	Oligodendrocyte precursor cells
ABA	Allen Brain Atlas
KEGG	Kyoto Encyclopedia of Genes and Genomes
GSEA	Gene Set Enrichment Analysis
LCM	Laser capture microdissection

PFC	Pre-frontal cortex
DE	Differential expression/Differentially-expressed genes
FAIR	Findable, Accessible, Interoperable, and Reusable
MADM	Mosaic analysis with double markers
MERFISH	Multiplexed error-robust fluorescence <i>in situ</i> hybridization
CL	The Cell Ontology
pCL	Provisional Cell Ontology

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ABSTRACT OF THE DISSERTATION

Distinct developmental trajectories and molecular signatures of layer II excitatory neurons in murine entorhinal cortex

by

Alanna Elizabeth Koehler

Doctor of Philosophy in Biomedical Sciences

University of California San Diego, 2022

Professor Robert F. Hevner, Chair

Entorhinal cortex (EC) cooperates with the hippocampal formation (HPF) in episodic memory encoding and spatial navigation, and it is one of the first human brain regions to degenerate in Alzheimer's disease (AD). EC layer II (L2) contains unique projection neurons, including Reelin⁺ stellate neurons (SNs) and clusters of Calbindin⁺ pyramidal neurons (PNs), that are selectively vulnerable to AD and remain poorly understood. Therefore, we aim to better characterize these neuron types with a focus on neurogenic trajectories and gene expression patterns.

Chapter 1 (Introduction) provides key background on the following: cortex organization; EC structure, function, and neuron types; classic “inside out” neocortical development versus current understanding of EC development; EC in disease; and transcriptional profiling of EC cells.

Chapter 2 is an original study into developmental timelines and trajectories of EC principal neurons, in which we address the hypothesis that some EC neurons undergo specialized migration and differentiation. Through thymidine analog birthdating, we demonstrate that murine EC diverges from classic “inside out” neocortical neurogenesis with a population of early-born L2 SNs (but not PN) that develops concurrently with deeper layers. We also dismiss the possibility precocious SNs may derive directly from early, potentially multipotent neuroepithelial cells (NECs) or radial glial cells (RGCs) by lineage tracing both PNs and SNs from neuron-fated intermediate progenitor cells (IPCs) despite their differing birthdates.

Chapter 3 details preliminary work in establishing a comparative atlas of EC neurons with the aim of better understanding (1) intrinsic properties that may influence differential disease susceptibility across neuron types and (2) functional significance of an early-born SN population. We perform single nucleus RNA sequencing (snRNAseq) on neuronally-enriched nuclei from murine and human EC, annotating clusters based on marker gene expression and laminar positioning. In a new collaboration with the Scheuermann Lab at J. Craig Venter Institute, we determine minimal marker genes that define each cell cluster, elucidating several new PN and SN markers. We also begin to probe differentially-enriched biological pathways between neuron types.

Finally, Chapter 4 provides a Summary and Future Work, including proposed experiments for understanding early SN development and plans for ongoing snRNAseq analysis.

CHAPTER 1:

Introduction of the Dissertation

1.1 CEREBRAL CORTEX ORGANIZATION

Laminar variation across regions of human cerebral cortex have been noted by anatomists since the beginning of the 20th century. The six-layered region comprising most of the cortex was termed “isocortex” for its homogeneity, while “allocortex” was used to describe regions with three-layers (one layer of cell bodies) believed to be more primitive (Vogt, 1903, 1910; translated by Judaš and Cepanec, 2010). Allocortex includes “archicortex,” or the hippocampal formation (HPF) consisting of dentate gyrus (DG), hippocampus proper (HP), and subiculum (SUB), and “paleocortex,” encapsulating piriform or primary olfactory cortex (Ariëns-Kappers, 1909). Mid-century, the transition region between isocortex and allocortex was further divided into two regions of “mesocortex” (Rose, 1926) (also called “juxtallocortex”), which includes the majority of limbic areas (Kaas, 2012; Ten Donkelaar et al., 2020). The term “proiscortex” refers to regions such as the perirhinal cortex, which are generally six-layered but not quite identical in patterning to isocortex (Bailey and von Bonin, 1951), and “periallocortex” refers to the transition between proisocortex and allocortex (Filimonoff, 1947), which encapsulates presubiculum (PrS), parasubiculum (PaS), and the region of most interest in this study: entorhinal cortex (EC) (Brod-

mann, 1909). This six-layered region is differentiated from isocortex and allocortex by a cell-sparse *lamina dissecans* in the position of layer (L) 4 (Insausti et al., 1995b, 1997b, 1997a, 2017; Burwell, 2001; Insausti and Amaral, 2008; Witter et al., 2017).

1.2 LOCATION, CONNECTIVITY, AND BASIC FUNCTION OF EC

Human EC corresponds to Brodmann's area 28 (Brodmann, 1909), situated in the parahippocampal gyrus of medial temporal lobe (MTL), where LEC shares a border with the collateral sulcus and, anteriorly, the rhinal sulcus. In rodents, EC can be found at the posterior extremity of the cortex, dorsolaterally bordered by the rhinal fissure and partially obscured by the cerebellum (Insausti et al., 1997b; Ray and Brecht, 2016; Kobro-Flatmoen and Witter, 2019). EC is histologically heterogeneous, with medial (MEC) and lateral (LEC) subdivisions recognized in rodents (Blackstad, 1956), and up to nine subdivisions recognized in primates, including humans (Amaral et al., 1987; Insausti et al., 1995).

In both primates and rodents, EC is positioned to serve as an interface between HPF and higher-order association cortices (Van Hoesen et al., 1972, 1975; Van Hoesen and Pandya, 1975a, 1975b; Van Hoesen, 1985). It is critical for spatial representation and navigation, and EC-HPF cooperation contributes to formation of declarative memories, including episodic memories involving spatial and temporal components (Van Hoesen, 1985; Buzsáki and Moser, 2013).

1.3 FUNCTIONAL EC NEURON TYPES

EC exhibits several neuron types, mainly defined in rodent models, that are unique in their molecular markers, clustering patterns, and/or firing patterns. However, there is little marriage between molecular and electrical classifications of these neuron types.

The majority of superficial EC neurons occupying L2/3 exhibit navigationally-based coding (Hardcastle, Maheswaranathan, Ganguli, & Giocomo, 2017). Electrophysiologically-defined grid cells in the superficial MEC fire in a fixed hexagonal pattern depending on location of the individual within an environment (Hafting, Fyhn, Molden, Moser, & Moser, 2005; Hargreaves, Rao, Lee, & Knierim, 2005; Hayman, Verriotis, Jovalekic, Fenton, & Jeffery, 2011; Kitamura et al., 2014). These spatially-modulated grid cells project via the perforant pathway to HP place cells (Hafting et al., 2005), which have individual “place fields” (O’Keefe, 1976) and integrate spatial and nonspatial inputs to create experience-specific representations (McNaughton, Battaglia, Jensen, Moser, & Moser, 2006; Moser, Kropff, & Moser, 2008). The superficial MEC is also home to speed cells, principal and interneurons which dynamically update representations of self-position in spatial maps (Carvalho et al., 2020; Iwase, Kitanishi, & Mizuseki, 2020; Kropff, Carmichael, Moser, & Moser, 2015; Sun et al., 2015; Ye, Witter, Moser, & Moser, 2018); head-direction cells, activity of which is modulated by direction of movement (Hafting et al., 2005; Sargolini et al., 2006); and sparse border cells, which fire along geometric borders of the environment (Sargolini et al., 2006; Savelli, Yoganarasimha, & Knierim, 2008; Solstad, Boccara, Kropff, Moser, & Moser, 2008). Recently, neurons within the LEC were found to respond to objects (Deshmukh & Knierim, 2011; Knierim, Neunuebel, & Deshmukh, 2014; Tsao, Moser, & Moser, 2013) in addition to their role in encoding temporal information across varying time scales—another fundamental element of episodic memory (Montchal, Reagh, & Yassa, 2019; Tsao et al., 2018). It has been proposed that these signals from the MEC and LEC collectively converge on the hippocampus to form a unique, integrated representation of experience, location, and time (Tsao et al., 2018).

Despite terms suggesting singular functions (i.e. “grid cells” or “border cells”), firing rates can be multiplexed within individual EC neurons to represent multiple variables of spatial navigation (Hardcastle, Maheswaranathan, Ganguli, & Giocomo, 2017). This property makes assigning functional cell type definitions to these neurons inherently difficult, and we turn instead to classification based on molecular markers or gene expression profiles to better understand heterogeneity within EC.

1.4 MOLECULAR EC NEURON TYPES

The largest proportion of grid-encoding cells is thought to reside in MEC L2 (Sargolini et al., 2006), which contains two principal neuron subtypes: distinct clusters, or “islands”, of pyramidal neurons (PNs) expressing Calbindin and *Wfs1*, surrounded by “oceans” of Reelin-expressing stellate (or fan) neurons (SNs) (Kitamura et al., 2014, 2015). Both PN and SN send axons to the HP via the perforant pathway. SNs project to the outer 2/3 of the granular layer of the DG and deep layer cornu ammonis (CA) 2/3 PNs (Tamamaki and Nojyo, 1993; Ye et al., 2018), while EC L2 PNs project to the stratum lacunosum of CA1 through the temporoammonic pathway (Kitamura et al., 2014; Ray et al., 2014; Ye et al., 2018).

Though they may have different inherent electrophysiological properties (Varga et al., 2010), both L2 PNs and, to some degree, SNs are capable of firing as grid cells (Tang et al., 2014; Sun et al., 2015). Border-encoding cells, on the other hand, tend to be L2 SNs, which provide the majority of spatial input to the DG (Tang et al., 2014; Sun et al., 2015). Despite this knowledge, compared to neocortex, for which there are extensive single cell atlases (Hodge et al., 2019), relatively little is known about individual EC neuronal subtypes—let alone their development and susceptibility to disease phenotypes.

1.4 OVERVIEW OF CLASSIC CORTICOGENESIS

We are interested in characterizing EC neuron types, including their developmental trajectories and timelines. Much work has focused on neocortical development, but the same attention has not been paid to mesocortical regions such as EC. The following is a primer on canonical neocortical neurogenesis:

Neurogenesis of glutamatergic neocortical neurons follows an “inside out” developmental gradient in which deep-layer excitatory neurons are born early in gestation and migrate a short distance along radial fibers to occupy laminae closer to the ventricular surface. Later-born neurons then migrate past earlier born neurons to upper cortical layers closer to the pial surface (Angevine and Sidman, 1961; Rakic, 1972).

Excitatory projection neurons occupying both deep and superficial layers are born from a similar cascade of neural progenitors. Early in gestation, (around embryonic day (E) 8 in mice) stem cells from the anterior neuroectoderm known as neuroepithelial cells (NECs) divide symmetrically to create an expanded pool of progenitors which, upon neural tube closure, span from the ventricular surface to the basal lamina of the pial surface (Smart, 1973; Radakovits et al., 2009). NECs gain glial characteristics to accompany their radial morphology, giving rise to a pool of Pax6⁺ radial glial cells (RGCs) that have both neurogenic and gliogenic potential (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Heins et al., 2002). RGCs undergo asymmetric divisions, allowing for self-renewal and the production of Tbr2⁺ (NCBI Gene: Eomes) intermediate progenitor cells (IPCs) (Miyata et al., 2001, 2004; Noctor et al., 2001, 2004; Haubensak et al., 2004). Tbr2⁺ IPCs are transit-amplifying cells committed to the genesis of projection neuron pairs, generally through terminal symmetric division away from the ventricular

surface in a distinct sub-ventricular zone (SVZ) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; Englund et al., 2005). Though a small number of neurons derive directly from either NECs or RGCs, the vast majority of neocortical projection neurons in all layers are produced from IPCs (Noctor et al., 2002; Malatesta et al., 2003; Anthony et al., 2004; Attardo et al., 2008; Kowalczyk et al., 2009; Mihalas et al., 2016; Mihalas and Hevner, 2018).

1.5 EC DEVELOPMENTAL PATTERNS

A recent study by Liu et al. (2021) identified 2 separate superficially-situated Reelin⁺ excitatory neuron populations in murine and porcine EC, characterized by the presence or absence of Ctip2/Bcl11b, generally a marker of deeper-layer neurons in neocortex (Arlotta et al., 2005; Chen et al., 2008). The presence of deep-layer neocortical markers in these upper-layer neurons calls begs the question: do SNs constitute a population of rare, early-born upper-layer neurons?

The suggestion of early-born L2/3 neurons in EC is not without precedent. Neuronal birthdating of periallo- and allocortical regions by tritiated thymidine revealed neurogenic patterns across the EC of several mammalian species, including mice (Angevine, 1965), rats (Schlessinger et al., 1978; Bayer, 1980), rhesus monkeys (Rakic and Nowakowski, 1981), and cats (Wyss et al., 1983). Several studies showed a mediolateral gradient of EC neurogenesis, with LEC developing slightly earlier than MEC (Angevine, 1965; Schlessinger et al., 1978), and, in a landmark paper, of rat hippocampal regions Bayer (1980) proposed a deviation from traditional “inside out” neurogenesis (Rakic, 1972) in EC. Bayer (1980) termed the EC-specific pattern a “sandwich gradient,” with L3 developing after neighboring L2 and L5 (across cell-sparse L4, or *lamina dissecans*). This “sandwich gradient” can be seen in subsequent diagrams of EC neurogenesis in

rhesus monkey by Rakic and Nowakowski (1981) and is clearly illustrated in feline LEC (Wyss et al., 1983).

The timeline of genesis for murine EC L2 neurons was investigated by Feng et al. (2015), who reported that many superficial neurons in MEC arise around E13.5, and EC neurogenesis is largely complete by E15.5. Recently, Liu et al. (2021) coined the term “parallel lamination” to describe the parallel genesis of L2 and L5/6 in mice. Importantly, the authors asserted that, unlike the “sandwich gradient” described by Bayer (1980) in rats, in which L3 neurons are born last, superficial L2 is still the last layer to complete neurogenesis in their mouse model despite the presence of some early-born neurons.

Notably, one of the first studies to birthdate murine EC neurons noted that the earliest born neurons in L2 appeared by Nissl staining be “star cells” (presumably SNs) (Angevine, 1965). In the last decade, groups have begun to examine differential developmental timelines of neuronal subtypes in EC L2. Two recent studies in mice observed Reelin⁺ SN genesis as early as E12.5, though they were restricted in timepoints analyzed (Donato et al., 2017; Liu et al., 2021).

Given the lab’s expertise in neurogenesis and neuronal birthdating, we sought to qualify these findings by expanding upon gestational ages sampled and independently validating the results with tamoxifen-inducible lineage tracing. We were also curious as to the neural progenitor(s) of origin given the slim yet present possibility that SNs could be a rare early-born neuron population deriving directly from NECs or RGCs without an IPC intermediate. While the expression of IPC marker Tbr2 has recently been documented in fetal pig EC (Liu et al., 2021), there has been little, if any, exploration of EC neural progenitors, especially as they relate to PNs and SNs.

1.6 EC IN DISEASE

The MT, including the EC-hippocampal formation (HPF) system, is prone to hyper-synchronization. Loop-gain amplification from parallel EC-HPF-EC loops and the possible development of recurrent excitatory synapses (Avoli et al., 2002; Kumar et al., 2007; Cataldi et al., 2013; Kuhn et al., 2018) or structural defects including specific neuronal loss (Du et al., 1993, 1995; Alhusaini et al., 2012, 2016) in the EC are potential causes of temporal lobe epilepsy (TLE), the most common form of focal epilepsy (Labate et al., 2011).

Unsurprisingly, damage to the MTL and EC have also been implicated in memory loss as early as the 1950s, when Milner and Scoville (1957) described a bilateral MTL resection in well-documented epilepsy patient Henry Molaison (known as H.M.) that resulted in severe retrograde amnesia affecting declarative memory (Scoville and Milner, 1957; Annese et al., 2014)

Most notable is the role of EC in the devastating neurodegenerative disease AD. Neurofibrillary pathology associated with AD begins in EC neurons of the transentorhinal cortex (a proisocortical transition point between temporal isocortex and periallocortical EC) (Braak and Braak, 1985; Insausti et al., 2017) before spreading to other limbic and wider isocortical association areas (Braak and Braak, 1991; Van Hoesen et al., 1991; Gómez-Isla et al., 1996).

L2 Reelin⁺ SNs (but not Reelin⁺ Cajal-Retzius cells in the marginal zone (Riedel et al., 2003)) are among the first cells affected in AD, followed by disruption of the perforant pathway and its terminal zone (Hyman et al., 1986, 1988; Van Hoesen et al., 1986; Arnold et al., 1991), possibly through transsynaptic propagation of misfolded tau (De Calignon et al., 2012; Harris et al., 2012; Liu et al., 2012). Previous observations indicate that L2 SNs accumulate hallmark neurofibrillary tangles (NFTs) and β -amyloid (β A) plaques even before significant neuronal loss occurs in AD brains (Hyman et al., 1984, 1986; Arnold et al., 1991; Kobre-Flatmoen et al., 2016).

Likewise, in a recent study, aged macaques with memory deficits exhibit a reduction in Reelin⁺ EC L2 neurons compared to age-matched controls (Long et al., 2020).

While most of the data point to a selective vulnerability of EC SNs, these findings are not consistent across the literature, with some studies showing profound loss of PNs and/or Calbindin expression in superficial EC associated with AD (Van Hoesen et al., 1986; Ichimiya et al., 1988; Iacopino and Christakos, 1990; Mikkonen et al., 1999; Thorns et al., 2001), or even a reduction of interneuron subtypes (Arai et al., 1987; Mikkonen et al., 1999; Riedel et al., 2003) that have been widely regarded as more resistant to degeneration (Hof et al., 1991, 1993; Iwamoto and Emson, 1991). These data are generated by candidate gene/protein analysis by immunohistochemistry (IHC) or single molecule fluorescence *in situ* hybridization (smFISH) rather than high throughput sequencing or ISH approaches. Such discrepancies highlight the need for unbiased studies into both cellular heterogeneity and selective vulnerability in EC.

Recent studies attempting to explore selective vulnerability in EC include Leng et al. (2021), which profiled >45,000 nuclei from EC of human donors of Braak stages between 0- 6 (higher stages indicate a greater extent of AD-related neurofibrillary pathology) (Braak and Braak, 1995). Leng et al. (2021) identified 9 excitatory neuron subtypes and described 3 selectively vulnerable populations, 2 of which were characterized by RORB expression and decreased synapse-related genes. It will be interesting to see if these results can be replicated and/or extended to determine if there is any relationship with Reelin⁺ SNs. Other similar studies utilizing snRNAseq in AD brains focus predominately on glial changes rather than selective vulnerability (Grubman et al. 2019).

1.7 SINGLE CELL RNA SEQUENCING OF EC NEURONS

The pioneering study for laminar-specific gene expression in murine EC is Ramsden et al. (2015), in which high throughput ISH is used for large-scale spatial analysis and identification of genes upregulated in EC. They compare expression to other brain regions, across EC layers, and at EC borders. Ramsden et al. (2015) proposes marker genes for all EC layers, including: *Dcc* (L2 SNs), *Wfs1* (L2 PN), *Kitl* (L3), *Etv1* and *Fezf2* (L5), and *Nxph4* (L6). To date, it also serves as the predominate reference for EC-specific gene expression.

Another key resource is a recent snRNAseq study across murine isocortex and HPF, that profile >65,000 neuronally-enriched EC nuclei (Yao et al., 2021). Yao et al. (2021) reinforce previous data showing that L2/3 EC neurons have profiles similar to deep-layer neocortical neurons (Donato et al., 2017; Liu et al., 2021) and incorporate spatial data to characterize 10 so-called “supertypes” of glutamatergic, intratelencephalic EC neurons by laminar position. Their results are further qualified by recent re-analysis with single cell assay for transposase-accessible chromatin with sequencing (ATACseq), which focuses on differences between MEC and LEC subregions (Blankvoort et al., 2022). These studies, in conjunction with Ramsden et al. (2015), provide useful marker genes for our analyses, and it will be critical to perform comparative analysis through dataset integration.

While efforts have been made to generate murine EC, atlases, very few groups have attempted a comprehensive single cell atlas of human EC. In a recent study comparing HPF neurons and neurogenic potential between several species, Franjic et al. (2022) profiled a relatively small number of EC nuclei from three human donors. EC neurons, however, were not the main focus of the paper, so, while the data uploaded to a public repository will be useful for analysis, the paper itself provided little specific insight into EC neuron types. We firmly believe a large, comparative atlas of human and murine EC neuron types with informative semantic ontology is

essential for better understanding this region, its peculiar neurogenic pattern, and its vulnerability to disease.

CHAPTER 2:

Distinct developmental trajectory of reelin⁺ stellate neurons in entorhinal cortex layer II

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2.1 ABSTRACT

Entorhinal cortex (EC) is a region of six-layered periallocortex that contains unique projection neuron types, including both Reelin⁺ stellate neurons (SNs) and Calbindin⁺ pyramidal neurons (PNs) in layer II (L2). EC exhibits distinct laminar organization from neocortex, marked by histological clusters of SNs and PNs in L2. Here, we studied layer-specific molecular markers and neurogenesis of EC neurons in mice to test the hypothesis that some EC neurons undergo specialized migration and differentiation. Unexpectedly, we found that L2 Reelin⁺ SNs expressed molecular markers typically associated with deep-layer neocortical neurons. Moreover, cell birthdating revealed that many Reelin⁺ L2 SNs were born between embryonic day (E) 10.5 to E12.5, ages typical of deep-layer neurogenesis in neocortex. In contrast, most L2 Calbindin⁺ PNs were born between E13.5 and 15.5, ages more typical of the birth of upper layer neurons. Genetic lineage tracing independently confirmed SNs and PNs are born at different ages and further showed that both SNs and PNs derived from Eomes⁺ intermediate progenitor cells (IPCs). These findings indicate that EC L2 Reelin⁺ SNs have many properties typical of deep layer neurons and may help explain why they segregate from Calbindin⁺ PNs within L2. The unique developmental history of L2 Reelin⁺ SNs may contribute to their exquisite vulnerability in Alzheimer's disease (AD), as they are the first cortical neurons to undergo neurofibrillary degeneration.

2.2 INTRODUCTION

Entorhinal cortex (EC), known in humans as Brodmann's area 28 (Brodmann, 1909), is a region of 6-layered periallocortex adjacent to the hippocampal formation. Regarded as one of the first brain regions to degenerate in Alzheimer's Disease (AD) (Ball, 1978; Braak and Braak, 1991, 1992, 1995; Van Hoesen et al., 1991; Gómez-Isla et al., 1996), EC is involved in spatial navigation and memory acquisition, and serves as an interface between the hippocampal formation (HPF) and higher-order association cortices (Van Hoesen et al., 1972, 1975; Van Hoesen and Pandya, 1975a, 1975b; Van Hoesen, 1985). EC-HC cooperation contributes to formation of declarative memories, including episodic memories (Van Hoesen, 1985), which have both spatial and temporal components (Buzsáki and Moser, 2013). In both humans and rodents, this region is anatomically distinct from neocortex, exhibiting conspicuous neuronal clusters and a unique lamination pattern with a cell-sparse *lamina dissecans*. Furthermore, EC is histologically heterogeneous, with medial and lateral subdivisions recognized in rodents (Blackstad, 1956), and up to nine subdivisions recognized in primates, including humans (Amaral et al., 1987; Insausti et al., 1995)

The two major excitatory projection neurons, as defined molecularly and anatomically, are Reelin⁺ stellate (or fan) neurons (SNs) and Calbindin⁺ pyramidal neurons (PNs), both of which populate EC layer II (L2) (Varga et al., 2010). In rodents, PNs form characteristic clusters, or “islands,” surrounded by “oceans” of unique Reelin⁺ SNs not found in neocortex (Kitamura et al., 2014, 2015; Sun et al., 2015; Ray and Brecht, 2016). Contributing to their distinctiveness, EC L2 neurons do not strictly follow the classic “inside out” neurogenic gradient demonstrated in neocortex, where deep-layer excitatory neurons are born early in gestation, while later-born neurons migrate to upper layers (Angevine and Sidman, 1961; Rakic, 1972). In contrast, some upper-layer EC neurons appear to be produced early in neurogenesis (Angevine, 1965; Bayer,

1980; Wyss et al., 1983; Donato et al., 2017; Liu et al., 2021). Previous groups have proposed “sandwich gradient” (Bayer, 1980) or “parallel lamination” (Liu et al., 2021) models of EC neurogenesis to describe the genesis of some EC L2 neurons before L3 neurons, and sometimes concurrently with L5/6. These previous studies did not, however, define the molecular profile, morphology, or neuron subtype differentiation of the divergent neuron population.

To understand the development of early-born L2 neurons, it is important to investigate the neural progenitor cell type(s) involved, as different progenitors may (in principle) be specialized to produce different neuron types. In neocortex, neuroepithelial cells (NECs) in the ventricular zone give rise to radial glial cells (RGCs), which, in turn, divide to produce intermediate progenitor cells (IPCs) (Miyata et al., 2001, 2004; Noctor et al., 2001, 2004; Haubensak et al., 2004). IPCs are a kind of transit-amplifying cell committed to neurogenesis of projection neurons and are distinguished molecularly by expression of transcription factor *Tbr2* (NCBI Gene: *Eomes*) (Englund et al., 2005). In neocortex, NECs and RGCs generate a small proportion of early-born neurons, while the majority of neocortical projection neurons in all layers are produced from IPCs (Haubensak et al., 2004; Kowalczyk et al., 2009; Mihalas et al., 2016; Mihalas and Hevner, 2018). *Tbr2* expression has previously been documented in fetal pig EC (Liu et al., 2021), but it remains unknown if EC L2 SNs and PNs derive from IPCs, RGCs, and/or NECs.

Our aim in the present study is to better characterize EC L2 neurons by elucidating possible molecular and neurogenic differences between the principal neuron types. We examined the expression of neocortical laminar markers in relation to PN and SNs, and then separately birthdated both populations. Further, we employed genetic lineage tracing and cell-type reporters to investigate the progenitor(s) of origin of these neuron types. We find that, while both PN and SNs derive from IPCs, they have distinct birthdates. These findings inform the segregation of

early-born and late-born neurons occupying the same laminar space and suggest possible underlying factors that influence the selective vulnerability of EC L2 neurons to neurofibrillary changes and degeneration in early AD.

2.3 MATERIALS AND METHODS

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of University of California San Diego and followed guidelines in the NIH Guide for the Care and Use of Laboratory Animals. Date upon which a copulatory plug was observed is defined as embryonic day (E) 0.5. Ages after birth are denoted as postnatal days (P). CD1 wild type mice were utilized for birthdating due to their robustness and large average litter size.

Eomes^{mTomatoH2B:GFP}(Eomes^{mTnG}) dual fluorescent reporter mice, as previously described (Probst et al., 2017), were maintained on a mixed Agouti background. mTomato is localized to the membrane of cells expressing Eomes, while H2B:GFP is targeted to the nucleus.

Previously-described hemizygous tamoxifen-inducible Eomes-Cre^{ERT2} (Pimeisl et al., 2013) mice on a C57BL6/J background were crossed with commercially-available homozygous Ai14 tdTomato reporter mice (Madisen et al., 2010) to generate lineage tracer mice. Tamoxifen and progesterone were co-injected at timepoints of interest, with progesterone used to combat the effects of tamoxifen-related toxicity and pregnancy complications (Fang et al., 1996; Gallo and Kaufman, 1997). Following injection, dams were sacrificed by cervical dislocation on E19.5 of pregnancy, and the uterine horn was removed for Caesarean section. Pups were freed from the uterine horn and amniotic sacs and warmed on a heating pad with agitation until consistent breathing and a dark pink skin color were observed. They were examined under a fluorescent

dissecting microscope for evidence of tdTomato red fluorescence in brain tissue, which can be visualized through the skull. tdTomato⁺ pups were placed with a foster dam that had produced a litter 1-2d prior and allowed to mature to age P7.5 or greater before sacrifice.

Injections

BrdU at 10 mg/ml working concentration was injected intraperitoneally into pregnant dams at a dose of 100 mg/kg. Tamoxifen (Sigma) was dissolved in corn oil (Thermo Fisher Scientific) at a concentration of 25 mg/ml and injected intraperitoneally at a dose of 50 mg/kg. Progesterone, also dissolved in corn oil, was co-injected with tamoxifen at a dose of 25 mg/kg.

Brain Fixation and Immunohistochemistry

Adult mice were anesthetized with 3% isoflurane, and intracardiac perfusion was performed using cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer. The brains were removed from the skull and allowed to postfix in 4% PFA at 4°C overnight before equilibrating overnight in 30% sucrose solution at 4°C. For 30 min, brains were equilibrated to a solution of 15% sucrose, 50% TissueTek OCT on ice, before being embedded in pure TissueTek OCT in an S-22 embedding mold (Polysciences Inc; 18646A-1) and flash frozen on dry ice. Tissues were then cryosectioned horizontally at 40 μ m, with free floating sections quickly transferred to ice cold 1x PBS in 24-well plates. For long-term storage, sections were transferred to cryoprotectant solution and stored at -20°C. Free floating sections were indirectly boiled in 10 mM sodium citrate, pH 6.0 for 8 min for antigen retrieval, and washed 3x in 1x PBS. If staining for BrdU, sections were incubated in 0.1 N hydrochloric acid at 37°C for 30 min for the purpose of DNA hydrolysis. Sections were washed a minimum of 5x in 1x PBS to neutralize pH before blocking in 10% normal

goat serum, 0.1% Triton X-100 (Sigma-Aldrich), and 2% bovine serum albumin (Sigma-Aldrich) for a minimum of 20 min at room temperature. Incubation with primary antibodies was performed overnight at 4°C, while secondary antibodies were applied for 2 h at room temperature. Sections were incubated in DAPI for 1 min and mounted to Superfrost Plus glass slides (Thermo Fisher Scientific). Glass coverslips were applied over Fluoromount-G (Thermo Fisher Scientific). Images were acquired with AxioVision software and a Zeiss AxioScope or Zen Black software with an LSM 780 confocal (Leica).

Primary Antibodies

Mouse anti-Reelin G10 (Millipore Sigma 5364; 1:1000), rabbit anti-Calbindin D28K (Swant CB38, 1:10000), mouse anti-Calbindin D28K (Millipore Sigma C9848; 1:2000), rat anti-BrdU (BioRad OBT0030; 1:250 or abcam ab6326; 1:1000), chicken anti-GFP (abcam ab13970; 1:500), rabbit anti-RFP (Rockland 600-401-379; 1:250), mouse anti-NeuN (Millipore MAB377, 1:1000), rabbit anti-Tbr2 (R.F.H., 1:1000), rabbit anti-Tbr1 (R.F.H; 1:000), rabbit anti-dsRed (ClonTech 632496; 1:1000), goat anti-Nurr1/NGFI-B beta/NR4A2 (R&D AF2156; 1:500), rabbit anti-Satb2 (abcam ab92446; 1:1000), goat anti-Foxp2 (Everest Biotech EB05226; 1:400); rabbit anti-Wfs1 (ProteinTech 26995-1-AP; 1:200), rabbit anti-PCP4 (Thermo Fisher PA5-52209; 1:1000), rat anti-Ctip2 (abcam ab18465; 1:2000), rabbit anti-Cdp/Cux1 (Santa Cruz 13024; 1:2000), mouse anti-Ror β (R&D PP-N7927-00; 1:200).

Cell Counting

Cell counting methodology was adapted from previous work (Hevner et al., 2004). Briefly, following fixation and immunostaining of 2 BrdU-injected murine brains per timepoint at P7.5, as

above, 20x Mosaix images were acquired of the entire EC (medial and lateral) using a Zeiss AxioScope compatible with image stitching in Zen Black software. Per staining combination and timepoint, images were taken for one hemisphere from each of 6- 40 μm horizontal sections evenly spaced from dorsal to ventral EC. Brightness and contrast of TIF images were adjusted in Adobe Photoshop 2022 (v23.2.2) for ease of counting. In a separate Photoshop layer, pial and ventricular margins were traced and 10 bins of equal thickness were imposed in between to generate a matrix. In a third layer, cells were marked by hand as either labeled with marker (Calbindin or Reelin) only, BrdU only, or both using color-coordinating dots. In Fiji (v20.0.0), the matrix bins were used to define regions of interest, and a custom macro was written to count dots of each color in every region of interest. Data was output to .csv files and integrated into a Pandas dataframe using Python (v3.8.8) for data management and graph generation with Matplotlib.

2.4 RESULTS

Murine EC is cytoarchitecturally/molecularly distinct from neocortex with L2 neurons expressing canonical “deep-layer” neocortical markers

Several studies have described the cytoarchitecture and lamination of mammalian EC, which is a 6-layered region of periallocortex with only 5 layers containing an abundance of cell bodies (L4, or the *lamina dissecans*, is cell-sparse) (Insausti et al., 1995, 1997b, 1997a, 2017; Burwell, 2001; Insausti and Amaral, 2008; Witter et al., 2017) (Fig 1A). In addition to its distinct lamination, the EC is also home to two unique excitatory projection neuron types residing in L2: (1) stellate (or fan) cells (SNs) that stain positively for Reelin, and (2) clusters of pyramidal neurons (PNs) that are predominately Calbindin⁺/Wfs1⁺ (Varga et al., 2010). We detect both cell populations in horizontal sections of fixed murine brain tissue, with Reelin and Calbindin antibody

reactivity clearly demarcating EC boundaries (Fig 1B). Wfs1 immunostaining also clearly shows L2 PN clusters (Fig. 1C). Other markers that delineate EC from neighboring neocortex and parasubiculum include Nurr1/Nr4a2, reportedly a L5 marker (Ramsden et al., 2015) but found at low levels in most laminae (Feng et al. 2021)(Fig. 1H), and Pcp4, which, in accordance with *in situ* hybridization (ISH) data from Ramsden et al. (2015), we find strongly labels L3 with fainter labeling of L5 (Fig. G).

In agreement with a recent study of porcine EC (Liu et al. 2021), we observed that classic markers of specific neocortical layers were expressed somewhat differently in EC. By double-label immunofluorescence, we observed that murine L2 Reelin⁺ SNs co-expressed Ctip2/Bcl11b, which is a marker of L5 subcerebral projection neurons in neocortex (Fig. 1D,I) (Arlotta et al., 2005; Chen et al., 2008). Furthermore, we discovered that the overwhelming majority of L2 Reelin⁺ SNs also expressed Tbr1, a marker of early-born glutamatergic neurons found at highest levels in L6 of neocortex (Fig 1F,J) (Bulfone et al., 1995; Hevner et al., 2001). Notably, there is no change in the expression pattern of Satb2, which plays a role in upper-layer neuron specification and marks excitatory neurons in all neocortical layers (Huang et al., 2013)(Fig. 1E). Together, these data indicate that L2 Reelin⁺ SNs in EC exhibit a molecular identity at least partially resembling deep-layer neurons in neocortex.

A portion of EC L2 cells are born concurrently with deeper layers

A landmark paper by Bayer (1980) reported the existence of early-born upper-layer neurons in rat EC by birthdating with tritiated thymidine, terming the neurogenic pattern a “sandwich gradient.” Bayer (1980) posited that EC L2 neurons arose concurrently with L5/6 neurons, and L3 neurons were born later. More recently, a report by Liu et al. (2021) confirmed a

similar pattern of neurogenesis in murine EC but introduced a different terminology, suggesting “parallel lamination,” in which some upper-layer neurons were born early, alongside L5/6 neurons—though L2 was still the last lamina to complete development.

Here, we sought to determine the distribution and identity of early-born neurons in upper layers of EC by birthdating with a thymidine analog (5-bromo-2'-deoxyuridine; BrdU). To analyze the laminar distribution of neuron cohorts, we plotted the locations of BrdU-labeled cells and performed bin analysis. Dams were injected with BrdU on different gestational days from E10.5 through E15.5, covering virtually all of mouse EC neurogenesis (Angevine, 1965). The BrdU-labeled cells were then counted in horizontal sections after survival to postnatal day (P) 7.5. These experiments confirmed that a small number of L2 neurons were born as early as E10.5 and E11.5, along with L6 neurons (Fig. 2). Neurons born on E12.5 (the peak of L5 neurogenesis) exhibited a distinct bimodal distribution in L2 and L5 (Fig. 2). A similar bimodal distribution was seen for neurons born on E13.5, although peaks now occupied L2 and upper L5/lower L3 (spanning the *lamina dissecans*). Most neurons born on E14.5 resided in L2/3, and neurogenesis was nearly complete by E15.5, when predominately L2 neurons were born (Fig. 2). These results confirmed that some—but not all—EC L2 neurons are indeed born concurrently with deeper layers.

Reelin⁺ SNs constitute the early-born population of L2 cells

We next investigated whether early-born EC neurons co-label with either Reelin or Calbindin, marking populations of SNs and PN, respectively. Calbindin⁺/BrdU⁺ neurons in L2 followed a typical neocortex-like, "inside-out" developmental sequence, with peak neurogenesis from E13.5-15.5 (Fig 3A). L2 Reelin⁺ SNs, however, were born as early as E10.5, and their neurogenesis continued through E15.5 (Fig 3B). Reelin⁺ and Calbindin⁺ neurons outside L2 were

likely GABAergic interneurons (Tremblay et al., 2016), although the possibility that these constitute presently uncharacterized excitatory neurons could not be excluded.

Both Reelin⁺ and Calbindin⁺ L2 EC neurons derive from Tbr2⁺ IPCs

Because the developmental timelines of Reelin⁺ and Calbindin⁺ neurons differed (Fig. 3), we hypothesized that these neuronal subtypes might derive from different progenitors, for example, with one subtype deriving from NECs/RGCs and the other from IPCs. To explore this possibility, we utilized 2 different mouse lines: (1) *Eomes*-driven membrane tomato, nuclear green fluorescent protein (GFP) dual fluorescent reporter mice (*Eomes*^{mTnG}), which facilitated short-term lineage tracing of neurons derived from Tbr2⁺ IPCs (Probst et al., 2017); and (2) *Eomes*-Cre^{ERT2} x Ai14 long-term lineage tracer mice, in which TdTomato (a red fluorescent protein) remains constitutively expressed in any neuron in which *Eomes* is actively transcribed into Tbr2 protein at the time of tamoxifen (Tx) injection (Pimeisl et al., 2013).

Labeling with *Eomes*^{mTnG} showed that most cells in the EC cortical plate during development from E12.5 to E14.5 were derived from Tbr2⁺ IPCs (Fig 4). Lineage tracing with *Eomes*-Cre^{ERT2} x Ai14 confirmed our conclusion from BrdU labeling that neurogenesis of L2 neurons began as early as E10.5 and continued through E15.5. Moreover, L2 neurons, including the early-born subset, were derived from IPCs as indicated by labeling with tdTomato in *Eomes*-Cre^{ERT2} x Ai14 mice (Supp. Fig 1). Analysis of TdTomato co-expression with Reelin or Calbindin indicated that both SNs (Fig 5) and PNs (Fig 6) were derived from IPCs, although SNs were the only subtype generated early in neurogenesis (before E13.5). The genesis of both SNs (early- and late-born) and PNs from IPCs was consistent with previous findings in neocortex, which

demonstrate that $\leq 10\%$ of cortical excitatory projection neurons derive directly from RGCs (Kowalczyk et al., 2009). In the present study, we did not quantitatively assess neurogenic divisions in EC, and so it remains possible that a subset of L2 SNs or PNs might be derived from NECs/RGCs.

2.5 DISCUSSION

In this work, we investigated the precocious genesis of some L2 neurons in EC and found that the early-born subset consists primarily of Reelin⁺ SNs, which are produced throughout EC neurogenesis from E10.5-E15.5 (Fig. 3). In contrast, Calbindin⁺ PNs, which constitute a second projection neuron type in EC L2, did not exhibit substantial neurogenesis until E13.5-E15.5 (Fig. 3). In addition, immunofluorescence detection of canonical laminar markers in EC demonstrated that L2 SNs (but not PNs) express genes characteristic of deep neocortical layers, despite their superficial positioning (Figure 1). Although their birthdays differed, at least a subset of both Reelin⁺ SNs and Calbindin⁺ PNs were derived from Tbr2⁺ IPCs (Figures 5 and 6). Thus, the relative segregation of SNs and PNs into distinct clusters within L2 is not the result of genesis from different progenitor types.

In mammals, EC is distinct in its cytoarchitecture, molecular profile, and projection neuron types. Its borders can be distinctly defined by several marker genes, including Nurr1/Nr4a2 (Feng et al., 2015) and Pcp4, as confirmed here (Fig. 1G,H). Importantly, EC L2 contains unique SNs and PNs. The PNs, “islands” of which project through the perforant and temporoammonic pathways to innervate interneurons of hippocampal field CA1 (Kitamura et al., 2014; Ray et al., 2014), express Calbindin and/or Wfs1 (Fuchs et al., 2016) in a pattern opposite to that of Pcp4 expression (Burwell et al., 2017) (Fig. 1B,C). Reelin⁺ SNs also project via the perforant pathway

but target a different part of the hippocampus, sending their axons primarily to the molecular layer of the dentate gyrus, as well as deep layers of CA2 and CA3 (Kitamura et al., 2014; Ray et al., 2014).

The SN and PN neurons in EC L2 may have additional underlying heterogeneity. A recent study by Liu et al. (2021) identified 2 separate Reelin⁺ excitatory neuron populations in mice and pigs, characterized by the presence or absence of Ctip2/Bcl11b, generally a marker of deeper-layer neurons in neocortex (Arlotta et al., 2005; Chen et al., 2008). The mice used in this study exhibited robust Ctip2 staining in EC L2, co-staining with the vast majority of strongly Reelin⁺ SNs (Fig. 2I). We further classify these Reelin⁺ SNs as expressing Tbr1, a transcription factor marker of early-born glutamatergic neurons that is highly expressed in L6 of neocortex (Bulfone et al., 1995; Hevner et al., 2001) (Fig 2J). We did not observe Calbindin⁺ PNs co-stained for either Ctip2 or Tbr1 (data not shown). These findings supported the conclusion that some SNs (but not PNs) are early-born despite their superficial laminar positioning.

The suggestion of early-born L2/3 neurons in EC is not without precedent. Using the incorporation of tritiated thymidine into dividing DNA *in utero*, several early studies examined the genesis of hippocampal regions of several mammalian species, including mice (Angevine, 1965), rats (Schlessinger et al., 1978; Bayer, 1980), rhesus monkeys (Rakic and Nowakowski, 1981), and cats (Wyss et al., 1983). Though several studies defined a clear mediolateral gradient of EC neurogenesis, with the lateral EC developing slightly earlier (Angevine, 1965; Schlessinger et al., 1978), it was not until the incisive study of rat hippocampal regions by Bayer (1980) that a discrepancy from traditional “inside out” neurogenesis (Rakic, 1972) was proposed for EC. Bayer (1980) termed the EC-specific pattern a “sandwich gradient,” with EC L3 as the last lamina to be produced. A hint of the “sandwich gradient” can be seen in subsequent diagrams of rhesus monkey

EC neurogenesis by Rakic and Nowakowski (1981) and is clearly illustrated in feline EC, predominately in lateral regions (Wyss et al., 1983).

The timeline of genesis for EC L2 neurons in mice was also investigated by Feng et al. (2015), who reported that many superficial neurons in medial EC arise around E13.5, and EC neurogenesis is largely complete by E15.5. Adding another interpretation, Liu et al. (2021) coined the term “parallel lamination” to describe the parallel genesis of L2 and L5/6 in mice. Importantly, the authors asserted that, unlike a “sandwich gradient,” in which L3 neurons are the last born, L2 is the last to complete neurogenesis, comprising both early- and late-born populations. Similar to the present study, Liu et al. (2021) reported that only a subset of L2 neurons are born early; however, they did not identify the early-born neuron subtype.

Our observations in the present study of EC supported the "parallel lamination" model, in which L2 develops last, over the "sandwich gradient" model, in which L3 develops last. It is of note, however, that the sandwich gradient was described in rats (Bayer, 1980) while the parallel gradient was described in mice (Liu et al., 2021), and the possibility of interspecies differences cannot be excluded.

Notably, one of the first studies to birthdate EC neurons in mice briefly mentioned that the earliest born neurons in L2/3 appeared to be “star cells” (presumably SNs) upon Nissl staining (Angevine, 1965). However it was not until the last decade that groups began to examine differential developmental timelines of neuronal subtypes in EC L2. Two recent studies in mice, though restricted in timepoints analyzed, observed Reelin⁺ SN genesis as early as E12.5 (Donato et al., 2017; Liu et al., 2021). Our data confirm and extend these results, as we are the first to show Reelin⁺ SNs born as early as E10.5 and to compare the birthdates of both Reelin⁺ and Calbindin⁺ neurons from E10.5 through E15.5 (Fig. 3).

Our work raises the question of why Reelin⁺ SNs localize in L2 despite having molecular features of deep-layer neurons, and despite many of them being early-born. One possibility is that the genesis of SNs is somehow coordinated with protracted development of the dentate gyrus, which is the main target of SN axons (Tamamaki and Nojyo, 1993; Varga et al., 2010; Kitamura et al., 2014; Ray et al., 2014). Indeed, DG neurogenesis never completely stops in mice, but is maintained into adulthood. Early-born SNs in the EC may perhaps be essential in pioneering the first projections to the embryonic DG on E19, when the DG is still only about half formed (Supèr and Soriano, 1994; Nelson et al., 2020). Later-born SNs would presumably augment the EC-DG projection throughout its subsequent growth. While this might explain why SNs are produced over a long period, it is unclear why they ultimately all reside in L2.

In terms of disease relevance, Reelin⁺ SNs have been reported to be selectively vulnerable to histopathological changes and degeneration in prodromal AD (Hyman et al., 1984, 1986; Arnold et al., 1991; Chin et al., 2007; Kibro-Flatmoen et al., 2016), in aged macaque monkeys with memory deficits (Long et al., 2020), in a McGill-R-Thy1-APP rat model of AD (Kibro-Flatmoen et al., 2016), and in numerous transgenic mouse lines overexpressing human *APP* or variant *MAPT* (Chin et al., 2007; De Calignon et al., 2012; Pooler et al., 2013, 2015; Booth et al., 2016). Reduction of Reelin expression in EC is also associated with age-related cognitive decline in rats (Stranahan et al., 2011). However, it is still unclear as to why this particular neuron population may be acutely sensitive to degeneration. Some groups have proposed a “First In, First Out” hypothesis for the loss of structural connections and/or cell bodies in aging and disease, suggesting the first neural structures to mature may be the first to degenerate (Stranahan and Mattson, 2010; Toga and Thompson, 2014; Yang et al., 2014; Fu et al., 2018). This idea has been challenged in normal human aging (Yang et al., 2014), and is only one putative factor of many that may influence

selective vulnerability (Fu et al., 2018). However, considering the neuronal population most susceptible to early AD develops precociously, a “First In, First Out” model is perhaps worth further study in neurodegenerative disease, especially as it applies to the EC. One area of interest may be the accumulation of DNA and/or cellular damage in this cell population with aging.

Because L2 SNs are unique in their neurogenic timeline, we considered the possibility that they might arise directly from Pax6⁺ RGCs rather than through Tbr2⁺ IPCs, as a small percentage of neocortical excitatory neurons are reported to do (Miyata et al., 2001; Haubensak et al., 2004). Recently, Liu et al. (2021) identified Tbr2⁺ IPCs in the porcine medial EC, with possible colocalization of Tbr2 and Reelin during later gestational ages (likely Cajal-Retzius neurons). However, to our knowledge, no group has previously examined EC neural progenitors in mice. To investigate the origins of SNs and PNs, we employed *Eomes* reporter and lineage tracer mice. The reporter mice demonstrated a high abundance of IPCs in the embryonic EC cortical plate, similarly to nascent neocortical regions (Fig. 4). Using *Eomes*Cre^{ERT2};Ai14 lineage tracer mice with tamoxifen injections at different ages, we found that both SNs and PNs derived from *Eomes*⁺ IPCs. Though we cannot rule out the possibility that some EC L2 neurons may derive directly from RGCs, our results demonstrated that both early-born and late-born SNs arise from IPCs (Fig. 5), as do PNs (Fig. 6).

Additional research will be needed to understand the intrinsic signaling pathways and/or external cues driving the genesis and migration of early-born SNs. It is also important to understand what cell intrinsic factors may possibly influence the selective vulnerability of L2 SNs over other principal neuron types in EC. We suggest that future single cell RNA sequencing studies particularly targeting EC neuronal subtypes will be critical in understanding this highly unusual neuronal population and its relevance to human disease.

2.6 ACKNOWLEDGMENTS AND FUNDING

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2.7 AUTHOR CONTRIBUTIONS

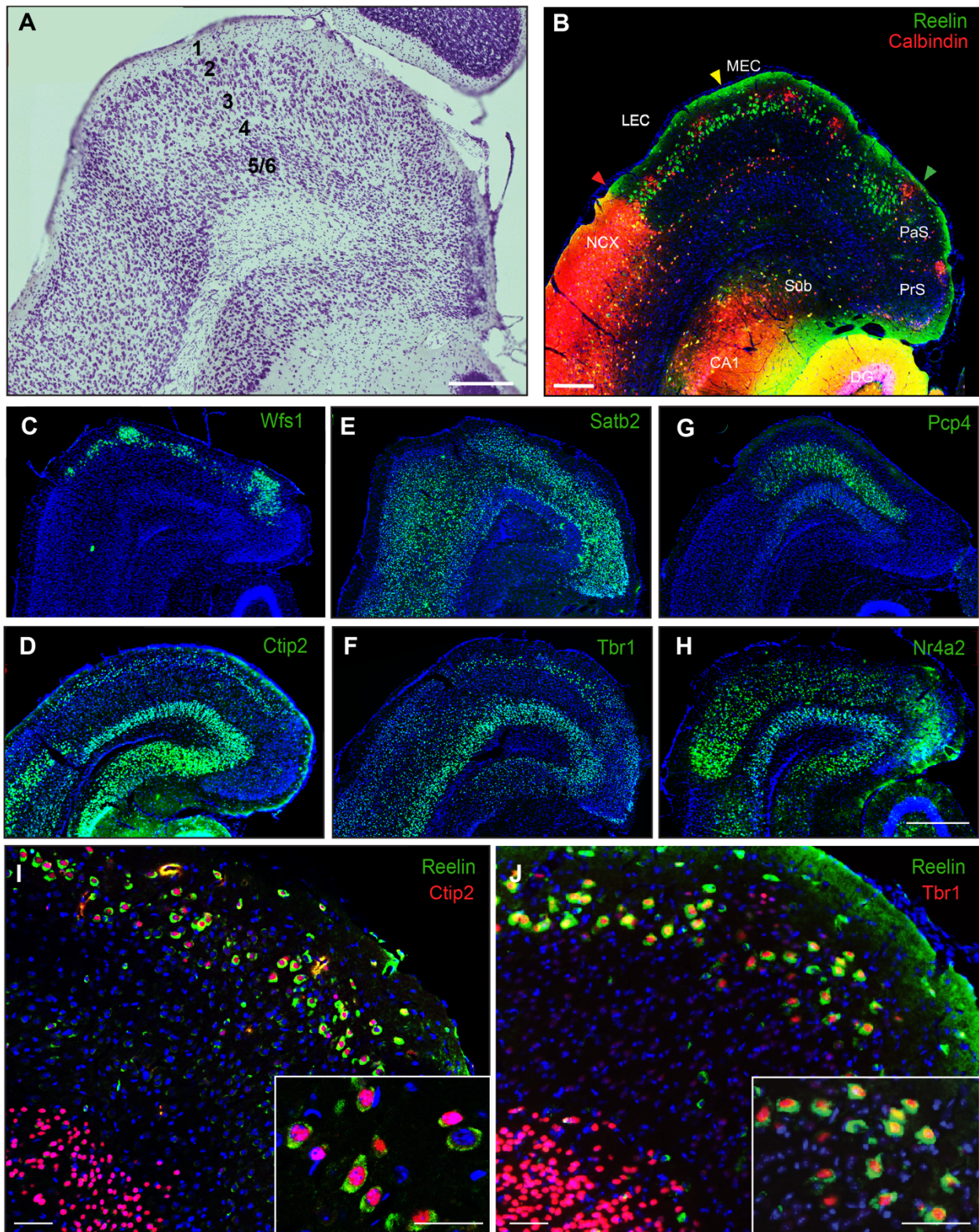
A.E.K.: Designed and executed experiments, analyzed data, and wrote manuscript.

R.A.M.D.: Assisted with experiments and manuscript editing.

R.F.H.: Conceived the project, obtained funding, designed experiments, and wrote manuscript.

2.8 FIGURES

FIGURE 2.1. Distinct cytoarchitecture and laminar distribution of molecular markers in murine EC. (A) Nissl stain of a horizontal section of a single hemisphere of caudal mouse brain, aged P7.5. EC laminae numbered 1 through 6. Note clusters of large-bodied neurons in EC L2 and a cell-sparse *lamina dissecans* constituting L4. Scale = 250um. (B) Immunostaining for molecular markers Reelin (green) and Calbindin (red) in a similar horizontal section of adult murine EC. LEC denoted from the red arrowhead to the yellow arrowhead and MEC from the yellow to the green arrowhead. Clusters of Calbindin⁺ cells can clearly be distinguished in L2, surrounded by Reelin⁺ neurons. Scale = 250um (C-H) Immunostaining for key laminar markers, including L2 marker Wfs1 (C), pan-excitatory neuron marker Satb2 (D), Pcp4 found at high levels in L3 (E), Ctip2/Bcl11b at high levels in L5/6 with additional staining in L2 (F), Tbr1 with L5/6 and L2 staining (G), and Nr4a2/Nurr1 staining L3 and 5 (H). Scale = 500um. (I-J) Co-immunostaining of Reelin (green) with Ctip2 (I) and Tbr1 (J) in EC L2. Insets show L2-specific co-localization. Scale = 50um; LEC = lateral entorhinal cortex; MEC = medial entorhinal cortex; NCX = neocortex; PaS = parasubiculum; PrS = presubiculum; Sub = subiculum; CA1 = Cornu Ammonis field 1; DG = dentate gyrus



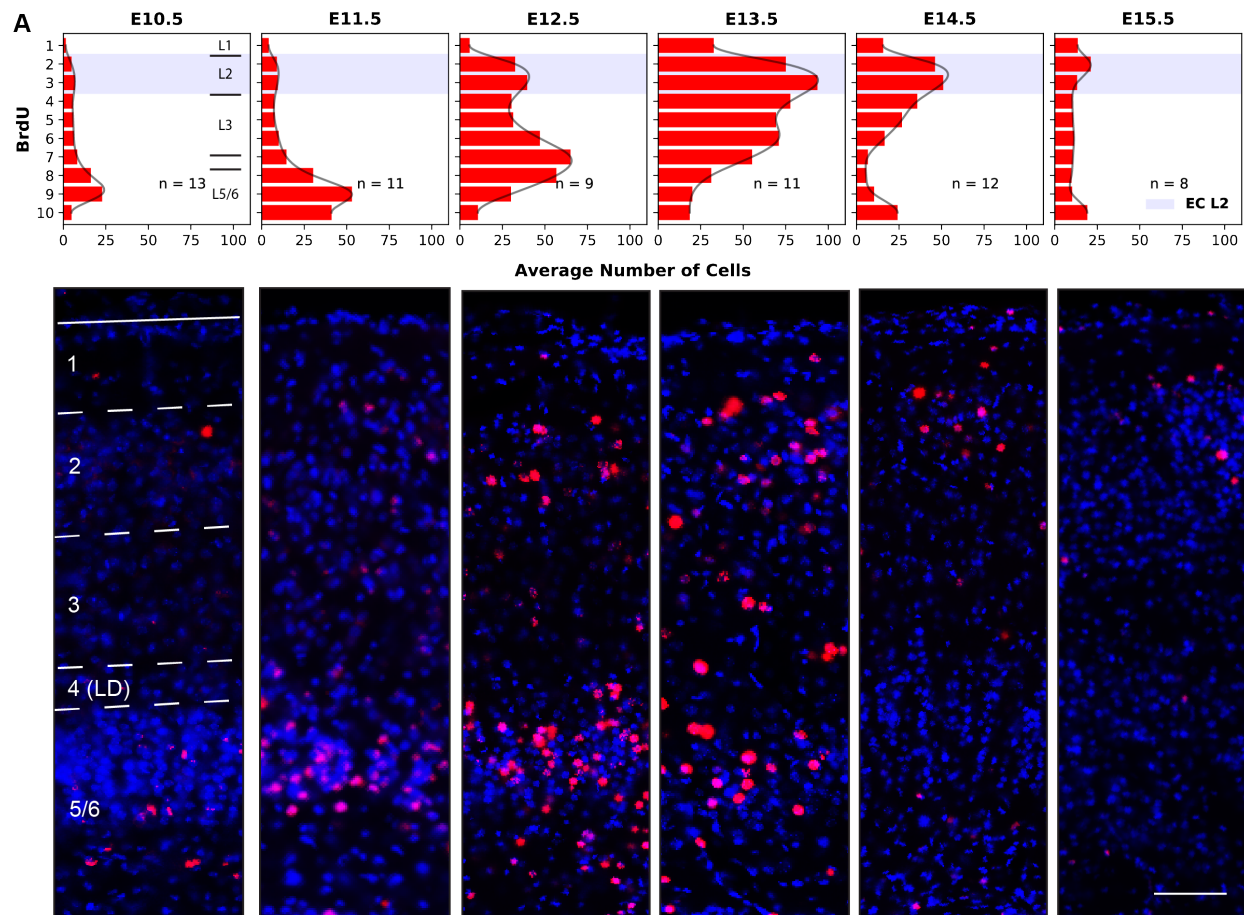


FIGURE 2.2. Birthdays and laminar positioning of cells in murine EC. (A) Histograms show the average number of cells in horizontal sections of P7.5 EC that incorporated BrdU on each embryonic day from E10.5 to E15.5. Bins are labeled from 1 (pial surface) to 10 (ventricular surface), with approximate location of EC L2 denoted by pale blue shading. Note that some L2 neurons were born concurrently with deeper-layer neurons, especially from E10.5-E12.5. All timepoints plotted on the same scale. (B) Representative BrdU immunostaining in horizontal sections of P7.5 mouse EC for each timepoint. Laminae and boundaries are labeled in the image corresponding to injection at E10.5. Scale = 50um; BrdU = 5-bromo-2'-deoxyuridine; LD = *lamina dissecans*

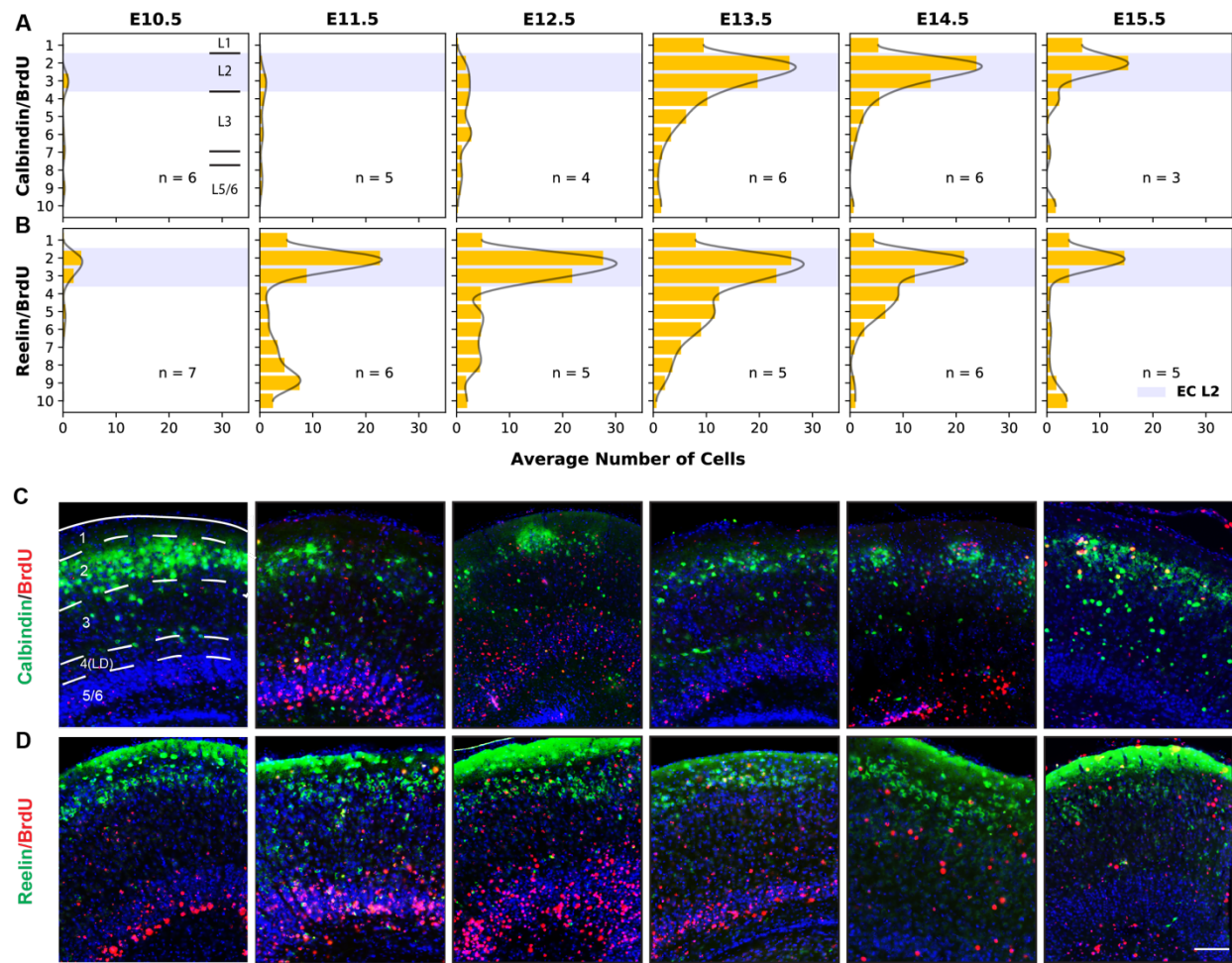


FIGURE 2.3. Birthdays and laminar position of Reelin⁺ SNs and Calbindin⁺ PNs in murine EC. (A-B) Histograms show the average number of Reelin⁺ (A) and Calbindin⁺ (B) neurons in horizontal sections of P7.5 EC that incorporated BrdU on each embryonic day from E10.5 to E15.5. Bins are labeled from 1 (pial surface) to 10 (ventricular surface), with approximate location of EC L2 denoted by pale blue shading. Note the early peaks in L2 Reelin⁺ SN neurogenesis but not Calbindin⁺ PN neurogenesis. All timepoints plotted on the same scale. (C-D) Representative immunostaining of BrdU (red) and Reelin (green) (C) or Calbindin (green) (D) in horizontal sections of P7.5 mouse EC, with BrdU injection days lined up beneath corresponding graphs in (A). Laminae and boundaries are labeled in (C) corresponding to injection at E10.5. Scale = 25um. BrdU = 5-bromo-2'-deoxyuridine; LD = lamina dissecans

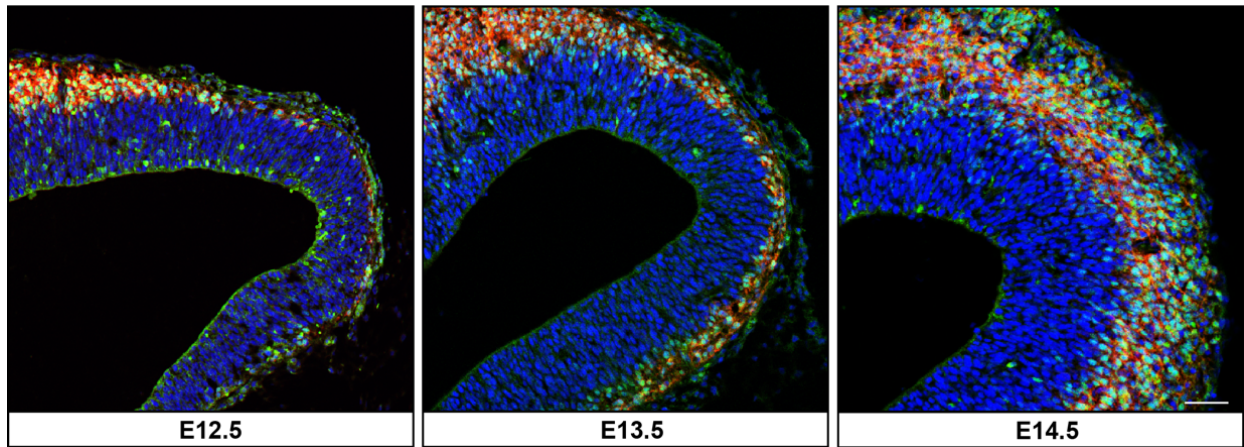


FIGURE 2.4. $Tbr2^{+}$ IPCs are present in embryonic EC and produce EC neurons. Horizontal sections of $Eomes^{mTnG}$ dual fluorescent reporter mouse brains at E12.5, E13.5, and E14.5. Neurons exhibiting red (membrane) and green (nuclear) fluorescence are derived from $Eomes^{+}$ IPCs. Scale = 20um

FIGURE 2.5. Lineage tracing and developmental timing of Reelin⁺ SNs derived from IPCs. Horizontal sections of Eomes-Cre^{ERT2} x Ai14 lineage tracer mouse brains at P21 injected with tamoxifen to induce constitutive production of TdTomato fluorescent protein at the following embryonic timepoints: E10.5 (**A-A'''**), E11.5 (**B-B'''**), E12.5 (**C-C'''**), E13.5 (**D-D'''**), E14.5 (**E-E'''**), and E15.5 (**F-F'''**). Single channel images shown for TdTomato expression (**A-F**) and Reelin expression (**A'-F'**). Merged TdTomato, Reelin, and DAPI shown in (**A''-F''**), with white boxes indicating areas enlarged in (**A'''-F'''**). Note that populations of L2 Reelin⁺ SNs in EC from IPCs as early as E10.5. Scale = 30um (20um for **A'''-F'''**)

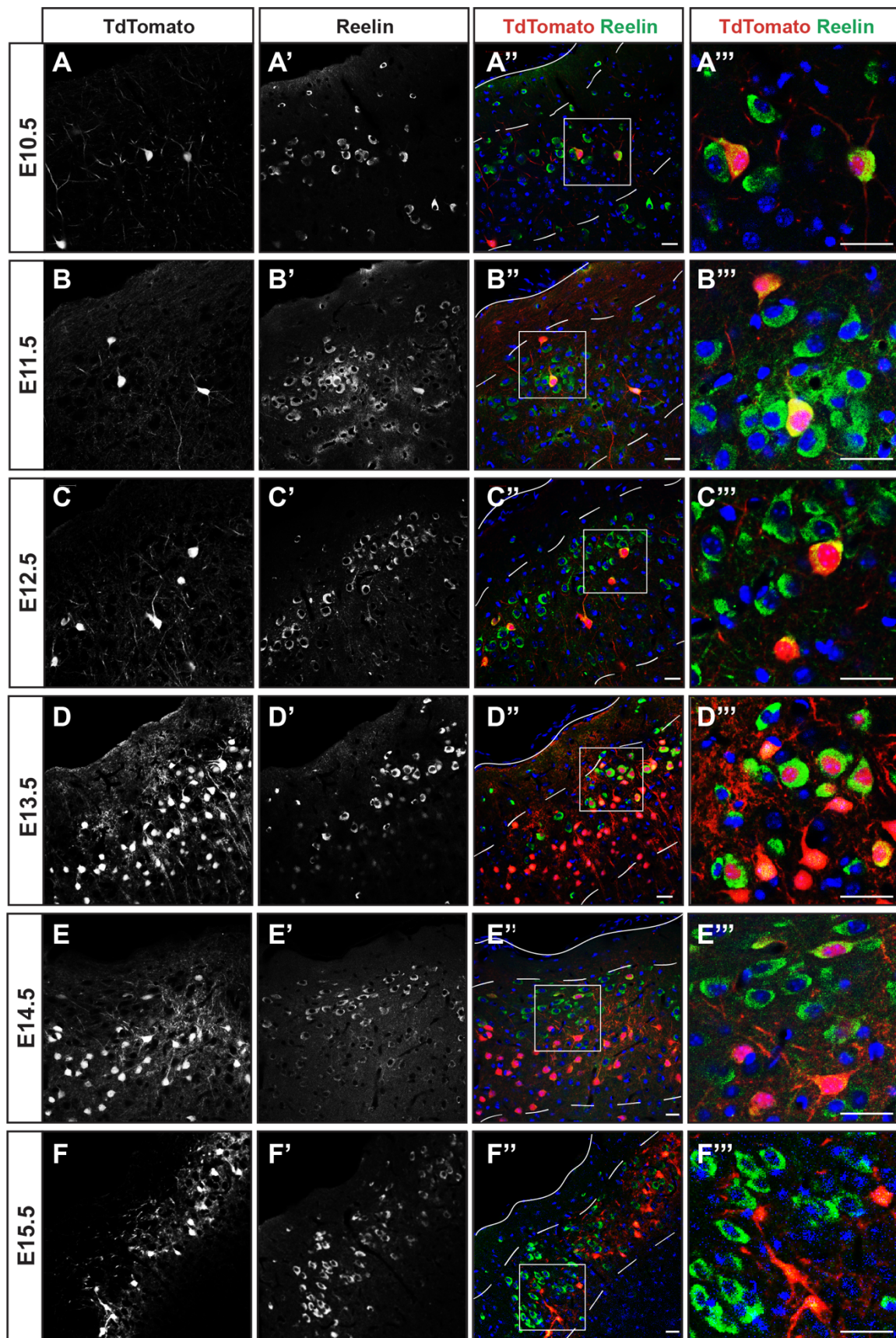
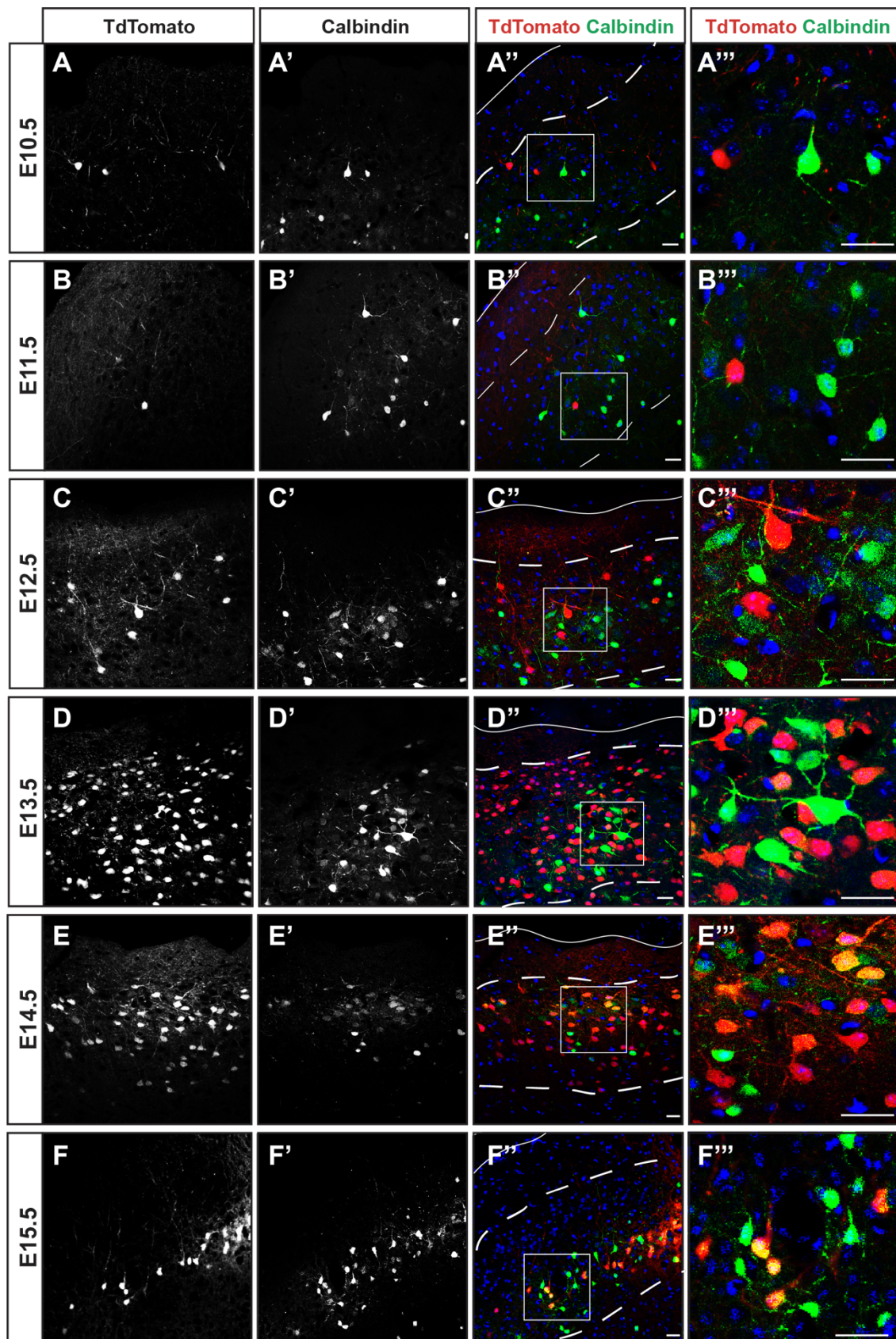
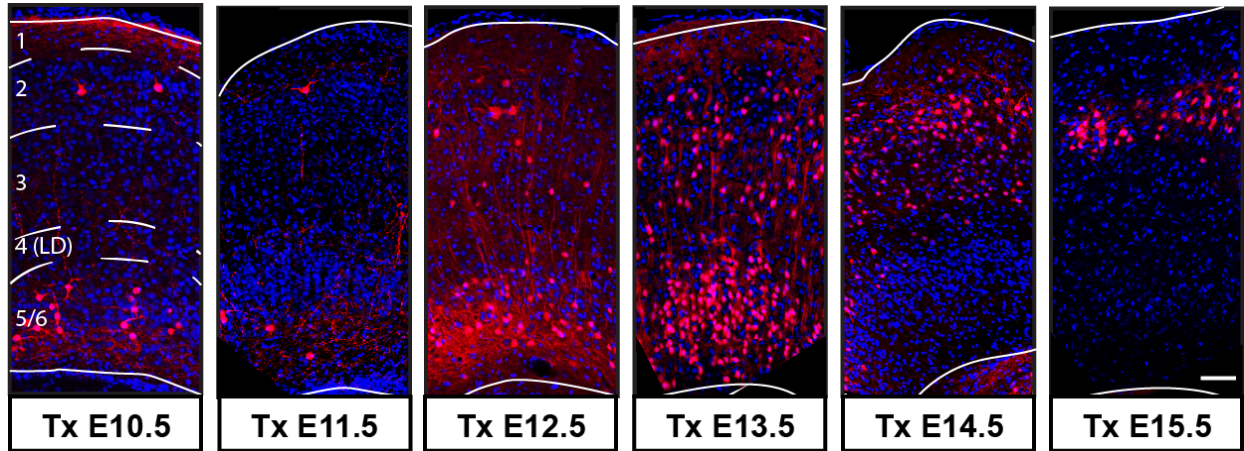


FIGURE 2.6. Lineage tracing and developmental timing of Calbindin⁺ PNs derived from IPCs. Horizontal sections of Eomes-Cre^{ERT2} x Ai14 lineage tracer mouse brains at P21 injected with tamoxifen to induce constitutive production of TdTomato fluorescent protein at the following embryonic timepoints: E10.5 (**A-A'''**), E11.5 (**B-B'''**), E12.5 (**C-C'''**), E13.5 (**D-D'''**), E14.5 (**E-E'''**), and E15.5 (**F-F'''**). Single channel images shown for TdTomato expression (**A-F**) and Calbindin expression (**A'-F'**). Merged TdTomato, Reelin, and DAPI shown in (**A''-F''**), with white boxes indicating areas enlarged in (**A'''-F'''**). Note that L2 Calbindin⁺ PNs are not generally born until later points in gestation. Scale = 30um (20um for **A'''-F'''**)



2.9 SUPPLEMENTARY INFORMATION



SUPPLEMENTAL FIGURE 2.7. Lineage tracing and EC neurons derived from IPCs. Horizontal sections of Eomes-CreERT2 x Ai14 lineage tracer mouse brains at P21 injected with tamoxifen (Tx) to induce constitutive production of TdTTomato fluorescent protein at the following embryonic timepoints: E10.5-E15.5. Layers are indicated by dashed lines and numbered. Margins are indicated by solid lines. Scale = 50 μ m.

Chapter 2, in full, will be submitted for publication of the material as it may appear in: Koehler, Alanna E.; Daza, Ray Anthony M.; Hevner, Robert F. Distinct developmental trajectory of reelin⁺ stellate neurons in entorhinal cortex layer II (2022) *Developmental Neurobiology*. To be submitted for review. The dissertation author was the primary investigator and author of this paper.

CHAPTER 3:

Transcriptomic analysis of neuronal subtypes in entorhinal cortex

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3.1 ABSTRACT

Entorhinal cortex (EC) is an under-studied, six-layered region of periallocortex with unique laminar organization and projection neuron types. The two principal neuron types in EC layer II (L2), including Reelin⁺ stellate neurons (SNs) and Calbindin⁺ pyramidal neurons (PNs), are defined by specific molecular markers and morphology. In previous work, we demonstrate that, despite occupying the same cortical layer, murine SNs and PNs diverge greatly in expression of canonical laminar markers and in neurogenic timeline. However, little is still known about their in-depth molecular signatures. Here, we used single nucleus RNA sequencing (snRNAseq) of NeuN⁺ murine EC neurons to investigate gene expression patterns and better understand the complexity of this region. We hypothesized a clear distinction between SNs and PNs, including differential expression of canonical laminar markers. From 33,506 NeuN⁺ nuclei passing quality control, we identified 28 distinct cell clusters, 23 of which represent neuronal populations. Cluster annotation allowed us to identify clusters of superficial (L2/3) and deep (L5/6) excitatory neurons, 4 broad categories of EC inhibitory interneurons, SNs, PNs, and a few small clusters of neighboring subicular (SUB) and hippocampal formation (HPF) neurons. We then utilized NS-Forest (v2.0) to identify minimal marker genes necessary and sufficient to define each neuron type. This technique elucidated several novel markers for PNs and SNs. Finally, we performed biological pathway analysis to parse out functional differences between key excitatory neuron population, and we delve into preliminary analysis of 7,373 human EC neurons.

3.2 INTRODUCTION

Entorhinal cortex (EC) is involved in spatial navigation and serves as an interface between the hippocampus (HP) and higher-order association cortices in the formation of declarative memories (Van Hoesen et al., 1972, 1975; Van Hoesen and Pandya, 1975a, 1975b; Van Hoesen, 1985). In both humans and rodents, EC is anatomically distinct from neocortex, with unique neuronal clusters and a cell-sparse *lamina dissecans*. Defined molecularly, the two major excitatory projection neurons of EC layer II (L2) are Reelin⁺ stellate (or fan) neurons (SNs) and pyramidal neurons (PNs) that express Calbindin and Wfs1 (Varga et al., 2010). PNs form characteristic clusters, also termed “islands,” that are surrounded by “oceans” of Reelin⁺ SNs (Kitamura et al., 2014, 2015; Sun et al., 2015; Ray and Brecht, 2016), and, despite occupying the same lamina, they exhibit differing neurogenic timelines. Reelin⁺ SNs constitute a divergent population of early-born, upper-layer neurons that express cortical markers associated with deep-layer neurons (Koehler et al., unpublished data; Donato, Jacobsen, Moser, & Moser, 2017; Liu et al., 2021) and have an acute susceptibility to neurofibrillary pathology and degeneration in early Alzheimer’s Disease (AD) (Hyman et al., 1984, 1986; Arnold et al., 1991; Chin et al., 2007; Kibro-Flatmoen et al., 2016).

SNs and PNs also differ in their connectivity and electrophysiological properties (Varga et al., 2010; Ye et al., 2018). Both PN and SN outputs exit EC and project via the perforant pathway to the hippocampal formation (HPF), which includes the dentate gyrus (DG), hippocampus proper or cornu ammonis (CA) fields, and the subiculum (SUB). SNs project to the outer 2/3 of the granular layer of the DG and deep layer CA2/3 PNs (Tamamaki and Nojyo, 1993; Ye et al., 2018), while EC L2 PNs project to the stratum lacunosum of CA1 through the temporoammonic pathway (Kitamura et al., 2014; Ray et al., 2014; Ye et al., 2018).

Electrophysiologically, EC is characterized by spatially-modulated grid cells that fire in a fixed hexagonal pattern (Hafting et al., 2005; Hargreaves et al., 2005; Hayman et al., 2011; Kitamura et al., 2014), along with speed cells (Kropff et al., 2015; Sun et al., 2015; Ye et al., 2018; Carvalho et al., 2020; Iwase et al., 2020), head-direction cells (Hafting et al., 2005; Sargolini et al., 2006), and border cells (Sargolini et al., 2006; Savelli et al., 2008; Solstad et al., 2008). Both L2 PN and SNs are capable of firing as grid cells (Sun et al., 2015), with PNs perhaps being more highly represented (Tang et al., 2014). Border cells tend to be L2 SNs, which provide the majority of spatial input to DG (Tang et al., 2014; Sun et al., 2015). A statistical approach, however, finds that the vast majority of EC cells exhibit mixed navigational coding (Hardcastle et al., 2017), suggesting electrical properties may not be the most defining characteristic of these neuronal subtypes.

To shed light on the various neuron types that constitute EC, as well as factors that may explain their varying birthdates and susceptibility to neurodegeneration, we turn instead to single nucleus RNA sequencing (snRNAseq). Compared to neocortex, for which there are extensive single cell atlases (Hodge et al., 2019), relatively little is known about individual EC neuronal subtypes. Most of our knowledge of the gene expression profiles across murine EC neurons comes from the study of high throughput *in situ* hybridization (ISH) (Ramsden et al., 2015) and a recent single cell atlas of Snap25-expressing murine isocortex and hippocampal formation neurons (Yao et al., 2021; Blankvoort et al., 2022). There are several published works that compare EC snRNAseq profiles from post-mortem samples taken from humans with and without clinical dementia, but many only profile a small number of nuclei and focus mainly on changes in glial signatures rather than specific neuronal subtypes (Grubman et al., 2019; Leng et al., 2021). We can also glean some insights into EC neuron diversity from recent Franjic et al. (2022), which

included transcriptomic profiles of EC nuclei from post-mortem human donors in a larger study of HPF cell types and neurogenic trajectories across species.

Here, we perform snRNAseq on 33,506 NeuN⁺ nuclei from murine EC using the 10x Genomics platform and classify glutamatergic and GABAergic neuron types based on Leiden clustering, gene expression, and predicted biological pathways. We utilize NS-Forest (v2), a Random Forest machine learning-based minimal binary marker gene selection algorithm previously described by Aevermann et al. (2021), for semantic ontology, and we explore functional Gene Set Enrichment Analysis for PN and SN annotated clusters. Finally, we perform preliminary clustering of a 7,373 NEUN⁺ nuclei from a post-mortem human EC sample as the first step in forming a comparative EC neuron atlas.

3.2 MATERIALS AND METHODS

Animals

All animal experiments were conducted on CD1 wild type mice and approved by the Institutional Animal Care and Use Committee of University of California San Diego. Experiments followed guidelines in the NIH Guide for the Care and Use of Laboratory Animals. Ages are denoted as postnatal days (P).

Tissue Harvesting for Murine snRNAseq

Mice were euthanized by cervical dislocation while incapacitated by 3% isoflurane gas. Brains were immediately dissected from the skull using RNase-free tools, cerebellum removed, and placed in ice cold, sterile-filtered 10 uM dithiothreitol (DTT; Sigma Aldrich; 43816) in 1x phosphate buffered saline (PBS; Thermo Fisher; J75889-K8). Brains were embedded in freshly-

prepared 4% low-melting agarose (BioExpress; E3121) in an S-22 embedding mold (Polysciences Inc; 18646A-1) and chilled to 4°C until cured. Blocks were then adhered to the stage of a Leica VT 1000S floating vibratome, submerged in a slurry of 10uM DTT-PBS and sectioned with a ceramic-coated blade at a thickness of 250 um. Sections were collected into NeuroTrace 500/250 Nissl Stain (Thermo Fisher; N21480) diluted 1:100 in DTT-PBS supplemented with a final concentration of 20 U/ul RNase inhibitor (Promega; N2615) and stained for a minimum of 5 min. Under an Olympus SZX16 fluorescent dissecting scope, the Nissl stain was used to locate cytoarchitectural boundaries. LEC and MEC were isolated together from successive sections and flash-frozen in liquid nitrogen and stored at -80°C.

Tissue Harvesting for Human snRNAseq

A freshly frozen human brain of a 73 year-old female donor was gifted from Joseph Gleeson. A section of EC was hand-dissected by neuropathologist R.F.H and stored at -80°C.

Nuclear Isolation and Staining

Dounce homogenizers and plungers were pre-incubated in nuclei isolation media (NIM) (0.25M sucrose, 25 mM KCl, 5mM MgCl₂, 10 mM Tris-HCl pH 7.5) with 25 mM DTT, 1x protease inhibitor (Sigma; NC0969110), and 0.5 U/ul RNasin (Promega; PAN2111) to make NIM-DP. Samples thawed on wet ice were resuspended in NM-DP with 0.1% Triton X-100 (Sigma; T8787) for lysis and dounce homogenized. Samples were then strained at 30um to remove debris and resuspended in NIM-DP. Nuclei were pelleted by centrifugation at 1000 rcf for 10 min at 4°C then rinsed once more with NIM-DP. After a second centrifugation, the supernatant was aspirated and the pellet resuspended in 500 ul Sort Buffer (1 mM EDTA, 0.5 U/ul RNasin, 1% BSA in PBS)

with mouse anti-NeuN monoclonal antibody clone A60, PE-conjugated (EMD Millipore; FCMAB317PE; 1:500) and incubated at 4°C for 30 min.

Nuclear Sorting

Stained nuclei were centrifuged at 500 rcf for 5min, washed with Sort Buffer, centrifuged a second time, and resuspended in Sort Buffer with DRAQ7 (Cell Signaling Technology; #7406S; 1:200). A total of 40,000 – 75,000 nuclei were sorted using the FANS gating strategy on a Sony Cell Sorter (Sony; SH800S) with Cell Sorter 100 μ M Chip (Sony; LE-B3001), and dual DRAQ7⁺/NeuN⁺ nuclei collected into low bind tubes with Collection Buffer (1 U/ul RNasin, 5% BSA in PBS). Nuclei were centrifuged at 1000 rcf for 15 min at 4°C, resuspended in Reaction Buffer (1 U/ul RNasin, 1% BSA in PBS), and counted on a hemacytometer using 0.4% Trypan blue (Fisher Sci; T10282) as a live/dead indicator.

Library Prep and Single Nuclei RNA Sequencing

A maximum of 18,000 nuclei were loaded onto a 10x Genomics Chromium Controller, and single nucleus GEM reactions and libraries were prepared using the Single Cell RNAs-seq 3' Reagent Kit v3 as per manufacturer's instructions. Samples were then sequenced by the University of California San Diego IGM Genomics Core to a target read depth of 50,000 reads/nucleus using the Illumina NovaSeq6000.

Bioinformatic Analyses

All computation was performed using the San Diego Supercomputer Center through the Triton Shared Computing Cluster (TSCC) at University of California San Diego

(<https://doi.org/10.57873/T34W2R>). Output FASTQ files for 10x Genomics 3'v3 snRNAseq were processed and aligned to the mm10 mouse reference genome or GRCh38 human reference genome using CellRanger software (v4.0.0; 10x Genomics). Data was merged using ScanPy (v1.5.1) (Wolf et al., 2018) in Python (v3.8) and an AnnData gene-count matrix was generated.

snRNAseq Quality Control and Normalization

The initial mouse dataset contained 36,810 nuclei and human dataset contained 8,750 nuclei. Cells with less than 1,000 counts or 500 genes were removed, as were cells with >20% mitochondrially-encoded genes or >5% ribosomal genes. For mouse data, one whole sample (JB_827) was eliminated from analysis due to a low number of recovered nuclei, high percentage of ribosomal genes, and low average gene counts per cell. In all samples, mitochondrially-encoded genes; hemoglobin genes; lncRNAs *Malat1*, *Gm42418*, and *4930467D21Rik* (due to incompatibility with NS-Forest); gene *AY036118*; and genes detected in less than 3 cells were excluded. The final datasets profile 23,157 genes across 33,506 nuclei and 26,544 genes across 7,373 nuclei, respectively (Table 1). Libraries were then normalized to a read depth of 10,000 reads/nucleus and the data logarithmized.

Cell Clustering

Using a reproduction of the Seurat (Satija et al., 2015) dispersion-based annotation method in Scanpy, we detected the top highly variable genes with a minimum dispersion of 0.5 for both mice and humans, resulting in 2,354 highly variable mouse genes and 4,138 human genes. The data was then 0-centered and scaled with a maximum value of 10. We computed principle component analysis (PCA) coordinates, loading, and variance decomposition using Scanpy's

implementation of scikit-learn (Pedregosa et al., 2011). We used graph-based clustering of 50 PCs and 15 nearest neighbors to compute neighborhood graphs that were then embedded in 2D using Uniform Manifold Approximation and Projection (UMAP) (Becht et al., 2018). We used the Leiden algorithm for community detection and graph-based clustering (Traag et al., 2019) and visualized the inter-relatedness of groups by dendrogram and Pearson correlation coefficient.

Cell Type Annotation

Clusters were annotated based on expression of marker genes from the literature, as described in the text. We assigned clusters to either excitatory neurons (Ex) (EC L2/3, EC L5/6, HPF), inhibitory neurons (In) (Pvalb, Sst, Vip, Reelin/Npy), and non-neuronal cells (NNCs) (astrocytes and ependymal cells, endothelial cells and pericytes, microglia, oligodendrocytes and oligodendrocyte progenitors (OPCs)). To help with annotation, we used Scanpy to rank genes for each cluster compared to the union of the rest of the groups using the Wilcoxon rank-sum test for P-value correction.

Marker Gene Identification

NS-Forest (v2.0), as previously described (Aevermann et al., 2021; Zhang et al., 2022), is a machine learning-based selection algorithm we used to identify minimal marker genes defining each cluster and to generate lists of binary marker genes akin to a cell-type barcode. Full result output can be found in Supp. Table 1.

Gene Set Enrichment Analysis.

We performed Gene Set Enrichment Analysis in Python (GSEAPy), a Python/Rust implementation of GSEA (Mootha et al., 2003; Subramanian et al., 2005) and wrapper for Enrichr (Xie et al., 2021) to explore Kyoto Encyclopedia of Genes and Genomes (KEGG) 2019 mouse PATHWAYS (Kanehisa and Goto, 2000) enriched in certain cell clusters with an adjusted P-value of <0.05 . We identified the top 5 enriched pathways per cluster of interest and plotted $-\log_{10}(\text{adjusted P-value})$.

3.3 RESULTS

Generation of a single nucleus transcriptomic atlas of murine EC neurons

To characterize specific EC neuron types, we dissected whole EC (including medial (MEC) and lateral (LEC) regions of both hemispheres) from 16 wild type C57Bl6/J mice and pooled samples into 5 batches for nuclear isolation and fluorescence activated cell sorting (FACS). We used the 10x Genomics Chromium platform based on single cell 3' version 3 chemistry to generate libraries of NeuN⁺, neuronally-enriched nuclei, which were then sequenced on the Illumina NovaSeq6000 to a target depth of 50,000 reads/nucleus (Fig. 1). After the quality control process, we obtained 33,506 high quality nuclear transcriptomes across 4 batches (Table 1). We visualized the relationships between nuclei with Uniform Manifold Approximation and Projection (UMAP) and inspected the distribution of sample batches (Fig. 2A). We then organized the nuclei into subgroups using the Leiden algorithm at a resolution of 0.6, which yielded 28 distinct clusters numbered from 0 to 27 (Fig. 2B). Based on the expression of known marker genes, including *Slc17a7* (glutamatergic neurons) and *Gad1* (GABAergic neurons), we divided the clusters into broad classes of excitatory neurons (17 clusters), inhibitory neurons (6 clusters), and non-neuronal cells (5 clusters), which were rare given our selection for NeuN⁺ nuclei (Fig. 2C). The separation

of these classes is easily visualized by a dendrogram representation of hierarchical clustering and corresponding Pearson correlation matrix (Fig. 2E).

We compared differential gene expression between clusters to gene sets from the literature that we cross-referenced to *in situ* hybridization expression patterns in murine EC from the Allen Brain Atlas of the developing mouse brain (ABA) (Lein et al., 2006). This allowed us to further divide our broad classes by function and, in the case of excitatory neurons, laminar positioning. We identified 6 subclasses of excitatory neurons, including 2 subclasses that putatively derive from neighboring HPF (Fig. 2F, Fig. 4). We show five inhibitory neuron subclasses, including 1 putative population of HPF interneurons, likely belonging to CA1 (Fig. 2F, Fig. 3). The remaining clusters are non-neuronal, with each of the 5 clusters representing its own cell subtype (Fig. 2F, Supp. Fig. 1).

GABAergic EC interneurons can be divided into MGE- and CGE-derived subtypes

GABAergic interneurons belong to two main lineages based on region of origin: those derived from the medial ganglionic eminence (MGE) and those from the caudal ganglionic eminence (CGE). MGE-derived interneurons include Sst and Pvalb subtypes driven by the following transcription factor cascade: Nkx2-1, Lhx6, Sox6/Satb1 (Liodis et al., 2007; Butt et al., 2008; Du et al., 2008; Azim et al., 2009; Batista-Brito et al., 2009; Close et al., 2012; Denaxa et al., 2012). Alternately, interneurons arising from CGE include Vip, Lamp5, Sncg, and Reelin (neurogliaform cells). CGE neurons can generally be lineage traced by Prox1 (Rubin and Kessaris, 2013; Miyoshi et al., 2015) and express Adarb2.

Here, we identify 2 MGE interneuron populations that express *Lhx6*, *Sox6*, and *Satb1*: Pvalb and Sst neurons, corresponding to clusters In5/24 and In8, respectively (Fig. 3A, B, E).

These annotations can likely be divided into further subpopulations with increased clustering granularity and/or nuclei number. For example, we see the suggestion of separate Pvalb-Tac1 (In5) and Pvalb-C1qlq (In24) subtypes by matrix plot expression data (Fig. 3E). When annotated, these MGE-derived subtypes cluster together by dendrogram, and we can compare expression of NS-Forest (v2.0) marker genes (Fig. 3F). Our Pvalb neurons are defined by combined expression of *Sox6*, *Btbd11*, and *Kcnc2*, while the lone Sst cluster is defined by high *Sst* and *Kcnc2*. *Kcnc2*, which encodes voltage-gated potassium channel subunit Kv3.2, is expressed in all detected interneuron types, with particularly high expression in MGE-derived types (Fig. 3F). *Btbd11* has recently been reported to be a synaptic scaffolding protein that supports excitatory synapses (Bygrave et al., 2021).

CGE neuron populations in our dataset include Vip/Calb2 and Reelin/Npy neurons (Fig. 3A). Clusters In5 and In10 express CGE markers *Prox1* and *Adarb2* (Fig. 3C, E), with In5 corresponding to Vip subtype GABAergic interneurons and In10 likely representing neurogliaform Reelin/Npy GABAergic interneurons in the Lamp5 lineage (Fig. 3A, E). Vip annotated neurons can easily be defined by binary expression of *Vip* alone, while *Egfr* and *Fgf13* expression define the Reelin subtypes (Fig. 3F).

Despite low *Gad1/2* expression and lack of MGE or CGE marker expression, In14 clusters closely to other GABAergic neurons (Fig. 3A, B, C, E) and is a presumed inhibitory neuron population. Given the expression of *Ntng1*, *Chrm2*, and *Htr2c*—known HPF genes—we label this cluster as putative Ntng1 HPF interneurons likely derived from neighboring CA1 (Fig. 3E). Our findings align with previous studies suggesting CGE-derived Ntng1 HPF interneurons do not express *Prox1* (Rubin and Kessaris, 2013; Miyoshi et al., 2015; Yao et al., 2021). This population

is defined by binary expression of transcription factor *Zfmx3* (Fig. 3F), which is enriched in DG and CA fields according to ABA.

A small number of non-neuronal cells, including glial and endothelial cells, could be detected

We detected a total of 1,363 non-neuronal cells (approximately 4% of total transcriptomes profiled) expressing the *Rbfox3* gene/NeuN protein after quality control (Fig. 2D). Of the 4 non-neuronal subclasses, we identify an endothelial cell and pericyte population encapsulating 2 clusters (Supp. Fig. 1A). This population expresses *Alcam* at high levels, which is a marker of blood brain barrier endothelial cells (Cayrol et al., 2007). We also find expression of several pericyte markers, including *Zic1*, *Pdgfrb*, and *Anpep* (Armulik et al., 2011) (Supp. Fig. 1B). We further detect 3 glial cell populations: oligodendrocytes (*Mbp*, *Mog*) and OPCs (*Sox10*), microglia (*P2ry12*, *Csf1r*), and astrocytes mixed with putative ependymal cells (Supp. Fig. 1A). This last cluster not only expresses canonical astrocyte markers (*Slc1a3*, *Gja1*), but also *Mt3*, found in ependymal cells (MacDonald et al., 2021), and *Aqp4*, which is found in both ependyma and the end feet of astrocytes (Nielsen et al., 1997) (Supp. Fig. 1B). Further exploration of these cell types is limited by cell number and beyond the scope of this study.

Clusters of excitatory glutamatergic EC neurons can be further defined by laminar positioning

EC excitatory neurons are far more numerous and complex than interneurons. We use a previous study of high throughput ISH signatures to subdivide EC glutamatergic neurons into superficial (L2/3) and deep (L5/6) subtypes, excluding cell body-sparse L4, or *lamina dissecans* (Ramsden et al., 2015). By plotting expression of L2/3 markers (*Cpne4*, *Epha3*, *Cux2*, *Trps1*) and L5/6 markers (*Tle4*, *Nfia*, *Zfp2*, *Sdk2*, *Foxp2*, *Pcsk5*, *Lmo3*, *Rai14*) (Fig. 4B,C,E), we identify

the following: 8 clusters of upper-layer neurons (Ex1, Ex3, Ex6, Ex7, Ex9, Ex11, Ex16, Ex22) and 5 clusters of deep-layer neurons (Ex0, Ex4, Ex17, Ex18, Ex20) (Fig. 4A). ISH data from ABA shows the laminar positioning of superficially-expressed genes *Cux2* and *Epha3* (Fig. 4G-G') and deeply-expressed genes *Tle4* and *Zfpm2* (Fig. 4H-H') in comparison to a Nissl stain annotated with all EC layers (Fig. 4F).

Four clusters do not fit neatly into this categorization, including Ex2, Ex13, Ex21, and Ex23 (Fig. 4E). Ex2 has characteristics most associated with L2/3 neurons, but may constitute a large, mixed population of cells for which further clustering granularity may provide clarity. The remaining three clusters all express *Tox* and *Etv1* (Fig. 4D, E), which are found not only in deep EC layers but also in SUB/HFP according to ISH data from ABA. Ex 23 highly expresses *Pde11a* and *Zbtb20*, while Ex13 and Ex21 express *Fnl*, *Serpine2*, and *Stxbp6* (Fig. 4E), suggestive of a HPF origin (Cembrowski et al., 2018; Lisi et al., 2019; Yao et al., 2021; Franjic et al., 2022).

Further work is needed to provide semantic cell type ontology each individual excitatory EC neuron type under our broad laminar categories. Toward that end, we provide NS-Forest (v2.0) marker and binary genes for each cluster (Table 2, Supp. Table 1), which will aid in the future characterization. Expression of minimal marker genes is shown for each excitatory cluster (Fig. 4I).

Distinct clusters corresponding to L2 SNs and PNs are discernible, allowing identification of novel marker genes

As SNs and PNs are key projection neuron types in AD-susceptible EC L2, we sought to identify both populations from other EC excitatory clusters by expression of known marker genes: *Wfs1*, *Calbindin*, and *Tshz2* for PNs and *Reelin* and *Bcl11b* for SNs (Fig. 5A). Based on expression

patterns across the 9 Ex L2/3 clusters, we assign Ex11 as PNs and Ex9 as SNs (Fig. 5B). Utilizing NS-Forest (v2.0), we identify binary genes characterizing these two cell types, including several novel marker genes. PNs are marked by known genes *Epha3*, *Megf11*, *Vwc2l*, *Tshz2*, *Wfs1*, and *Cacna2d3* (Ramsden et al., 2015; Blankvoort et al., 2022), and novel marker genes *Meis2*, *Gm20754*, *Gm26883*, and *Cdc14* (Fig. 5C, D). SNs are marked by known SN genes *Ephb1*, *Gpc5*, *Inpp4b*, *Reelin*, and *Dcc* (Ramsden et al., 2015), as well as novel marker genes *Fam19a2*, *Chsy3*, and *Gm36251* (Fig. 5C,E). Note there are no overlapping binary genes between the two, suggesting fundamental differences in cellular pathways and signaling. ISH data from ABA shows the L2 expression patterns of select PN (Fig. 5G-G'') and SN (Fig. H-H'') marker genes compared to an annotated Nissl stain of the same region (Fig. 5F). Note the distinct clustering of PNs in L2.

SNs are enriched for genes involved in energy production and disease processes

We performed Gene Set Enrichment Analysis (GSEA) on PNs and SNs and compared the $-\log_{10}(\text{adjusted P-value})$ between the top 5 enriched pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) 2019 mouse database for each population (Fig. 5I). While PNs were preferentially enriched in genes corresponding to Axon guidance (adj. P-value $5.853466\text{e-}09$), Glutamatergic synapse (adj. P-value $1.216937\text{e-}06$), Aldosterone synthesis and secretion (adj. P-value $1.364421\text{e-}06$), Circadian entrainment (adj. P-value $2.486397\text{e-}06$) and Oxytocin signaling pathway (adj. P-value $8.365559\text{e-}06$), SNs were enriched for the following: Synaptic vesicle cycle (adj. P-value $5.352608\text{e-}07$), Thermogenesis (adj. P-value $3.534253\text{e-}06$), Oxidative

phosphorylation (adj. P-value 5.849364e-06), Huntington disease (adj. P-value 1.702518e-05), and Alzheimer disease (adj. P-value 2.985658e-05).

Towards building a comparative atlas of human EC neurons

A future goal is to build a comparative atlas of human EC neurons from postmortem human tissue of control and AD patient brains. We dissected a region of frozen, unfixed human EC and performed snRNAseq on NEUN⁺ neuronal nuclei following the same protocol used for mouse samples. After quality control, our yield was 7,373 nuclear transcriptomes from one 73 year-old female donor. We have begun visualizing relationships between the data with UMAP and annotating clusters generated with the Leiden algorithm (granularity 0.8) (Supp. Fig. 3A). We examine expression of excitatory neuron (*SLC17A7*), inhibitory neuron (*GAD1*), and non-neuronal markers to separate the clusters into broad cell type categories (Supp. Fig. 3B,E) that cluster together by dendrogram and Pearson correlation coefficient (Supp. Fig. 3D, E). Our yield of neurons is >89% with just over 10% non-neuronal cells. We are currently further annotating clusters to generating a cell type specific ontology, and preliminary results for inhibitory neuron gene expression profiles (Supp. Fig. 3F) and excitatory neuron gene expression profiles (Supp. Fig. 3G) are given. Based on this information, we assigned the clusters to coarse cell types, including putative HPF excitatory neurons, L2/3 excitatory neurons, L5/6 excitatory neurons, CGE-derived inhibitory neurons, MGE-derived inhibitory neurons, putative HPF inhibitory neurons, and non-neuronal cells (Supp. Fig. 3C).

3.4 DISCUSSION

In this study, we compile a snRNAseq atlas of murine EC that is enriched for neurons (~96%) (Fig. 1,2). We identifying canonical interneuron subtypes, which previous literature shows vary little between cortical regions (Tasic et al., 2016; Yao et al., 2021) (Fig. 3), and focus on EC lamina-specific glutamatergic neuron subtypes (Fig. 4). In addition to providing novel binary marker genes for each neuronal cluster, we also identify and characterize populations of L2 SNs and PNs from gene expression to biological pathway enrichment (Fig. 5). Finally, we introduce a preliminary snRNAseq atlas of human EC neurons (~89%) to be expanded and further annotated in the future (Supp. Fig 3). We are working towards comparative analysis and development of a semantic cell type ontology for EC neurons of both species to aid in understanding the molecular makeup of this region and to help inform selective vulnerability of certain EC neurons to disease processes.

Comparison to Previous High Throughput Gene Expression Studies

The landmark study for laminar-specific gene expression in murine EC is Ramsden et al. (2015), which used high throughput ISH for large-scale spatial analysis and identification of genes upregulated in EC compared to other brain regions, across EC layers, and at EC borders. It proposes marker genes for all EC layers, including: *Dcc* (L2 SNs), *Wfs1* (L2 PNs), *Kitl* (L3), *Etv1* and *Fezf2* (L5), and *Nxph4* (L6). To date, it also serves as the predominate reference for EC-specific gene expression, including for this study.

Another key resource is a recent snRNAseq study across murine isocortex and HPF, including profiles of 62,308 EC neurons and another 1,594 and 1,564 profiles from LEC and MEC, respectively, from SNAP25-IRES-Cre x Ai14 mice (Yao et al., 2021). Yao et al. (2021) reinforce previous data showing that L2/3 EC neurons have profiles similar to deep-layer neocortical

neurons (Koehler, unpublished data; Donato et al., 2017). They also characterize 10 so-called “supertypes” of glutamatergic, intratelencephalic EC neurons by laminar position using marker gene ISH and Visium spatial transcriptomics. Their results are further qualified by recent re-analysis with single cell ATAC-seq, which focuses on differences between MEC and LEC subregions (Blankvoort et al., 2022). These studies provide a baseline for our analysis and will be useful for future dataset integration and comparison.

In a large study comparing HPF neurons between several species and tracing species-specific neurogenic signatures, Franjic et al. (2022) profiled single nuclei from EC of three human donors. They plotted gene expression of several marker genes and assigned a cell type ontology based on laminar positioning and marker genes. They also generated a large dendrogram of the HPF-EC system in humans, and showed that some SUB neuron types cluster with EC neurons. Though the single cell profiles will be useful for comparative analysis between datasets, EC neurons are not the main focus of the paper. In fact, there are few studies that have attempted a comprehensive single cell or nucleus atlas of the human EC. Most focus on changes in cell states and gene expression between control brains and those from AD patients. For example, Grubman et al. (2019) profiled a little more than 13,000 nuclei from control and AD human brains with most of the cells belonging to glial subtypes. In a more extensive study, Leng et al. (2021) profiled 45,528 nuclei from post-mortem human EC, with subjects of Braak stage 0, 2, and 6 indicating the extent of neurofibrillary pathology. They identified 9 excitatory neuron subpopulations and described 3 selectively vulnerable populations (2 characterized by RORB and 1 with high CDH9 expression) with downregulated synapse-related genes. This gene down-regulation was corroborated by microarray data from laser capture microdissection (LCM) of neurons from six regions of post-mortem AD brains (Liang et al., 2008) and a recent pre-print looking at prefrontal

cortex (PFC) neuron susceptibility to AD (Marinaro et al., 2020). Their transcriptomic atlas was compared to an atlas of PFC neurons between AD and control brains (Mathys et al., 2019) to compare cell types and vulnerability. They also compared laminar marker gene expression to Ramsden et al. (2015). In our dataset, *Rorb* serves as a binary marker gene for Ex3 (Supp. Table 1).

In addition to EC neurons, we identify several putative populations of HPF and/or SUB neurons (Fig. 2F) that are likely small contaminants from neighboring regions captured during microdissection. In addition to neocortical reference atlases, we also consulted studies on various regions of HPF (Thompson et al., 2008; Cembrowski et al., 2016a, 2016b; Bienkowski et al., 2018; Ayhan et al., 2021; Yao et al., 2021) and SUB (Cembrowski et al., 2018; Ding et al., 2020) to identify regional marker genes. The Allen Brain Atlas for developing mouse brain (ABA) (Lein et al., 2006) also provided an invaluable resource for visualizing ISH signal for binary marker genes corresponding to putative HPF populations, including, for example, *Serpine2* in CA1.

We are currently work on integrating our single nuclei transcriptome data with the above described datasets to generate a better consensus of cell type ontology. It will also be worth comparing to neocortical single cell atlases (Lake et al., 2016; Tasic et al., 2016; He et al., 2017; Hodge et al., 2019; Yuste et al., 2020) to compare and contrast neuron types and gain a deeper understanding of the paradoxical deep-layer marker gene expression in superficial L2 SNs and PNs.

Novel SN and PN Marker Gene Identification

EC L2 SNs and PNs are unique for their clustering patterns, molecular markers, and differing neurogenic timelines (Kitamura et al., 2014, 2015; Sun et al., 2015; Ray and Brecht, 2016; Donato et al., 2017). EC L2 is also one of the first regions of the human brain to degenerate in AD (Ball, 1978; Braak and Braak, 1991, 1992, 1995; Van Hoesen et al., 1991; Gómez-Isla et al., 1996), with differential susceptibility to neurofibrillary pathology and degeneration reported between the 2 principal neuron types (Hyman et al., 1984, 1986; Arnold et al., 1991; Chin et al., 2007; Kibro-Flatmoen et al., 2016). Therefore, they are of particular interest in our analyses.

Based on expression of *Calbindin*, *Wfs1*, and *Tshz2*, we label cluster Ex11, corresponding to a population of L2/3 glutamatergic neurons, as PNs (Fig. 5A,B). In addition to known marker genes reported by Ramsden et al. (2015) and Blankvoort et al. (2022), we identified the following novel marker genes by NS-Forest (v2.0): *Meis2*, *Gm20754*, *Gm26883*, and *Cdc14* (Fig. 5 C,D; Supp. Table 1). *Gm20754* and *Gm26883* are long non-coding RNAs (lncRNAs), adding credence to the notion that lncRNAs show high cell-specific expression patterns (Djebali et al., 2012). *Meis2*, also a marker gene for Ex0 (a deep-layer EC cluster), is a three amino acid loop extension (TALE) homeobox protein responsible for transcriptional activation of *Epha8* (Shim et al., 2007), an ephrin receptor which, in ABA, is also lightly expressed in EC L2. According to NCBI, *Cdc14* is a phosphatase involved in cell cycle regulation.

We and other previously report that SNs express deep-layer neocortical markers, including *Bcl11b*, also known as *Ctip2* (Koehler et al., unpublished data; (Liu et al., 2021). Based on expression of *Reelin* and *Bcl11b*, we label EC L2/3 cluster Ex9 as SNs. Aside from known marker genes *Ephb1*, *Gpc5*, *Inpp4b*, *Reelin*, and *Dcc* (Ramsden et al., 2015), we report 3 novel marker genes with binary expression: *Fam19a2*, *Chsy3*, and lncRNA *Gm36251* (Supp. Table 1). *Fam19a2*,

also known as Tafa2, is a small, secreted chemokine that may play a role in immune cell regulation according to NCBI. Its expression has been reported in EC L2 (Yao et al., 2021; Blankvoort et al., 2022) but, to our knowledge, not specifically in PNs. *Chsy3*, also known as *Css3*, encodes chondroitin sulfate synthase, a glycosyltransferase (Yada et al., 2003). A single nucleotide polymorphism (SNP)-SNP interaction between gene pair *CHSY3-GALNT2* (glycan structure biosynthesis) is implicated in EC atrophy (Meda et al., 2013). It is our hope that these markers will not only aid in cell type population identification but, with more advanced understanding of protein function, perhaps inform cellular process specific to different neuron subtypes. Understanding differential biological pathways upregulated in susceptible versus robust neurons will be crucial to the understanding and development of therapeutics for diseases like AD.

To preliminarily address this, we performed GSEA analysis of KEGG mouse pathways in SNs and PNs. We plotted the $-\log_{10}(\text{adjusted P-value})$ of the top 5 pathways identified for both clusters to compare enrichment between groups. While PNs were enriched for pathways involved in axon guidance and glutamatergic synapse function, SNs were enriched for disease pathways, including AD, as well as thermogenesis and the synaptic vesicle cycle (Fig. 5I). Additional work is needed on the functional implications of these findings and whether they influence selective vulnerability to degeneration.

The Value of Binary Marker Genes for Semantic Ontology

A major goal of this work is to determine marker gene combinations for each cluster that capture EC neuronal identities in a reproducible and informative manner and to eventually contribute to semantic representations of each that are compliant with FAIR principles for scientific data management (i.e. Findable, Accessible, Interoperable, and Reusable) (Wilkinson et

al., 2016). To this end, we utilize NS-Forest (v2.0) (Aevermann et al., 2021) to elucidate minimal marker genes and generate binary “barcodes” to help define each cell type. NS-Forest is a machine-learning algorithm that utilizes random forest feature selection and binary expression scoring, differing from classic differential expression analysis (DE) used by Seurat (Stuart et al., 2019) and Scanpy (Wolf et al., 2018) that employ rank-sum testing without providing a readout of genes with classification power (Aevermann et al., 2021).

We will submit finalized cell type taxonomies to a Cell Ontology database (Diehl et al., 2016; Osumi-Sutherland et al., 2021) for cell types that are defined by single cell transcriptomics. The aim of the field should be an atlas of annotated, canonical cell types that can be bolstered with additional data on cell phenotype, including morphology and electrophysiology.

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3.7 AUTHOR CONTRIBUTIONS

A.E.K: Designed and executed experiments, analyzed data, and wrote manuscript.

Y.Z.: Analyzed data and wrote manuscript.

N.T: Assisted with analysis.

R.A.M.D: Assisted with experiments.

R.H.S.: Helped to conceptualize project, analyzed data, and wrote manuscript.

R.F.H.: Conceptualized project and wrote manuscript.

3.8 FIGURES

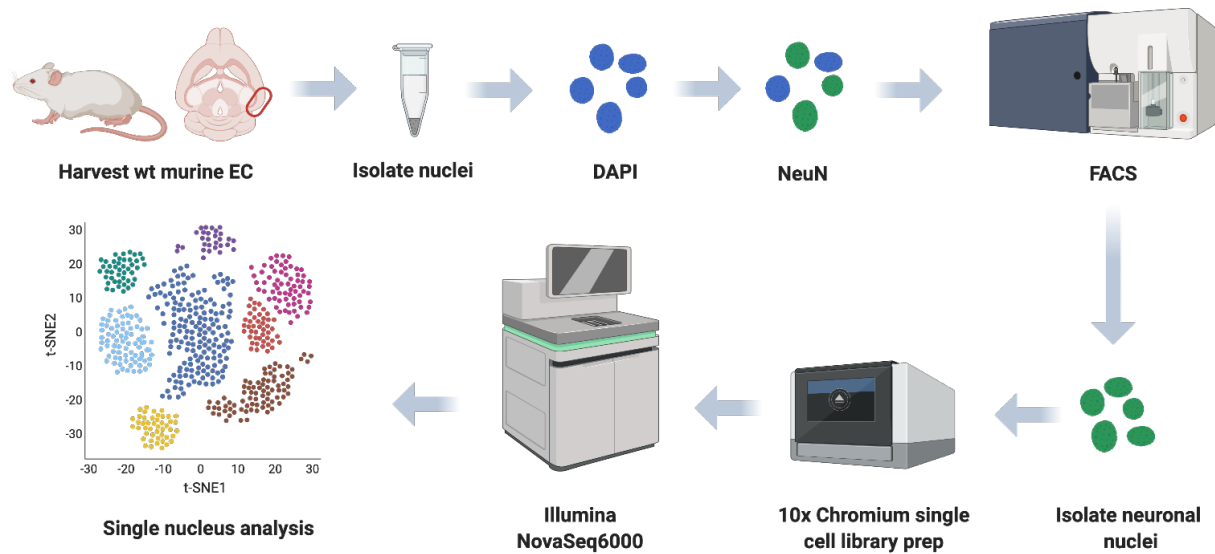
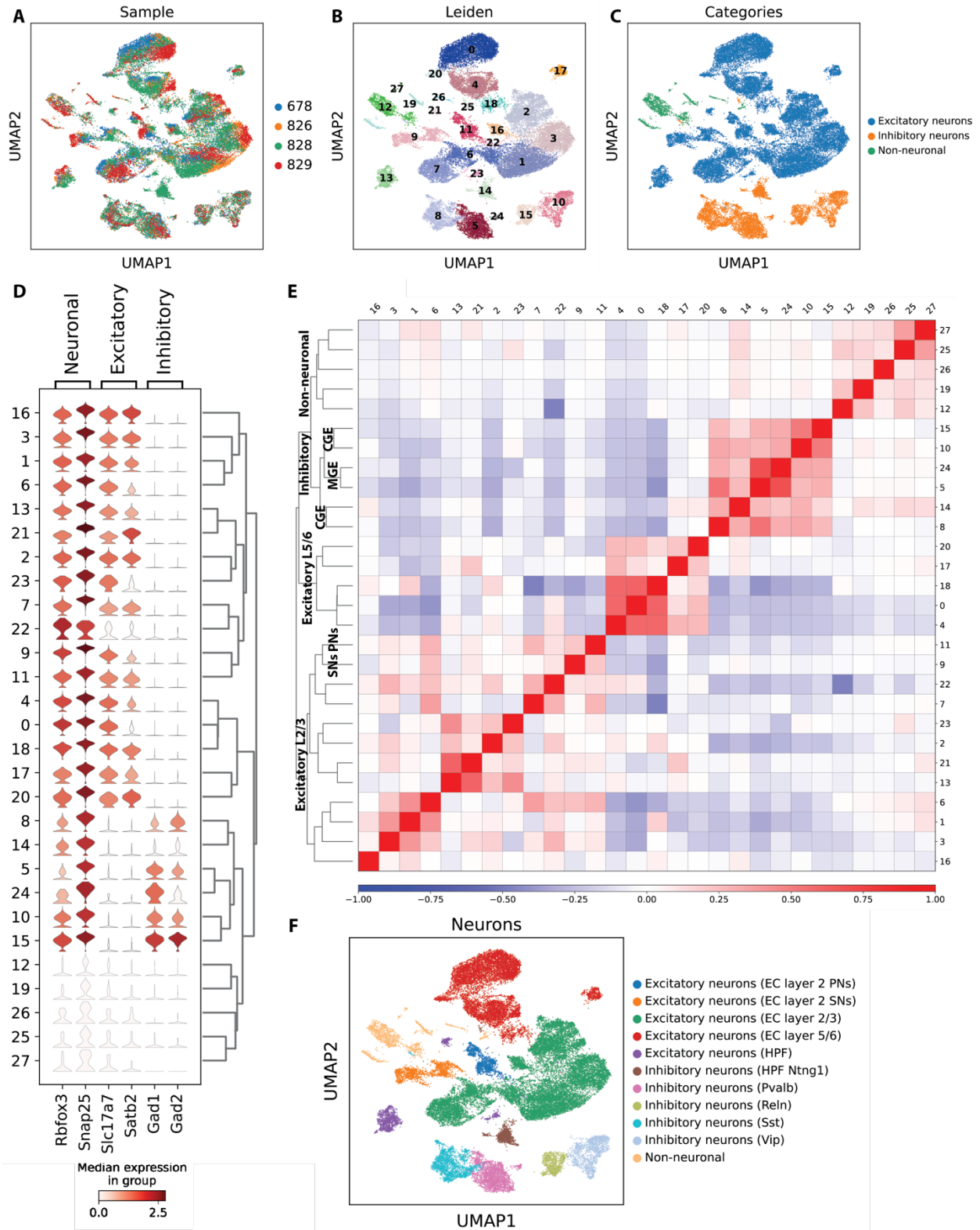


FIGURE 3.1. Workflow for tissue harvesting, nuclear isolation, and snRNAseq. Briefly, the EC was microdissected from horizontal sections of wild type mouse brain and nuclei were isolated by density centrifugation after Dounce homogenization. Samples were incubated with DRAQ7 dye and anti-NeuN antibody to stain neuronal nuclei, and dual-positive nuclei were collected by FACS. Single cell libraries were prepared by 10x Genomics RNA 3' v3 on the Chromium Controller and sequenced on the Illumina NovaSeq6000 for downstream analysis. Generated using BioRender.com.

FIGURE 3.2. Sample clustering and annotation of murine nuclei. (A-C) UMAP representation of single nuclei labeled by sample (A), Leiden clustering and numbering (B), and broad classification into non-neuronal cells, excitatory neurons, and inhibitor neurons (C). (D) Dendrogram of relatedness between clusters accompanying a stacked violin plot of raw expression data of basic neuronal marker genes. Color intensity represents average expression level of each gene per cluster, while the shape shows the distribution of cells with high or low expression. (E) Correlation plot displaying the Pearson correlation coefficient for each cluster as compared to all other clusters as they are ordered by dendrogram. (F) UMAP representation of annotated neuron types and non-neuronal cells.



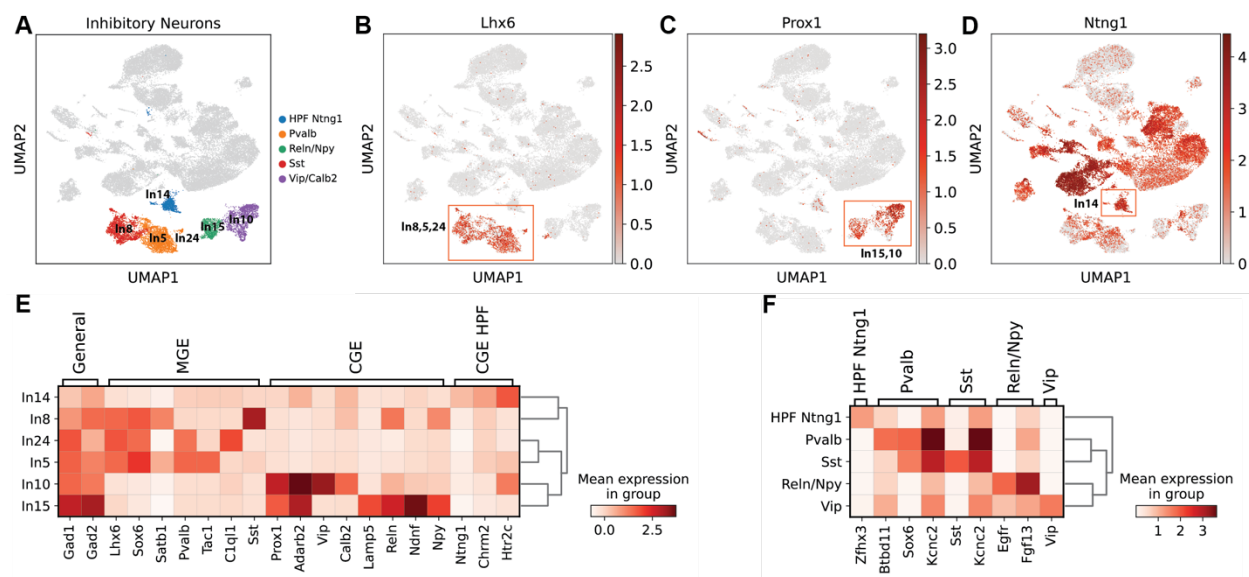


FIGURE 3.3. GABAergic neuron types. (A-D) UMAP representation of annotated inhibitory neuron classes (A) and marker gene expression for *Lhx6* corresponding to MGE-derived interneurons (B), *Prox1* corresponding to CGE-derived interneurons (C), and *Ntng1* corresponding to interneurons of the HPF. Clusters corresponding to these cell types are boxed. (E) Dendrogram of relatedness between inhibitory (In) clusters with a matrix plot of key marker gene expression. (F) Dendrogram clustering of annotated interneuron types and matrix plot of expression levels of identifying marker genes discovered using NS-Forest (v2.0). Color intensity in matrix plots represents average gene expression per cluster

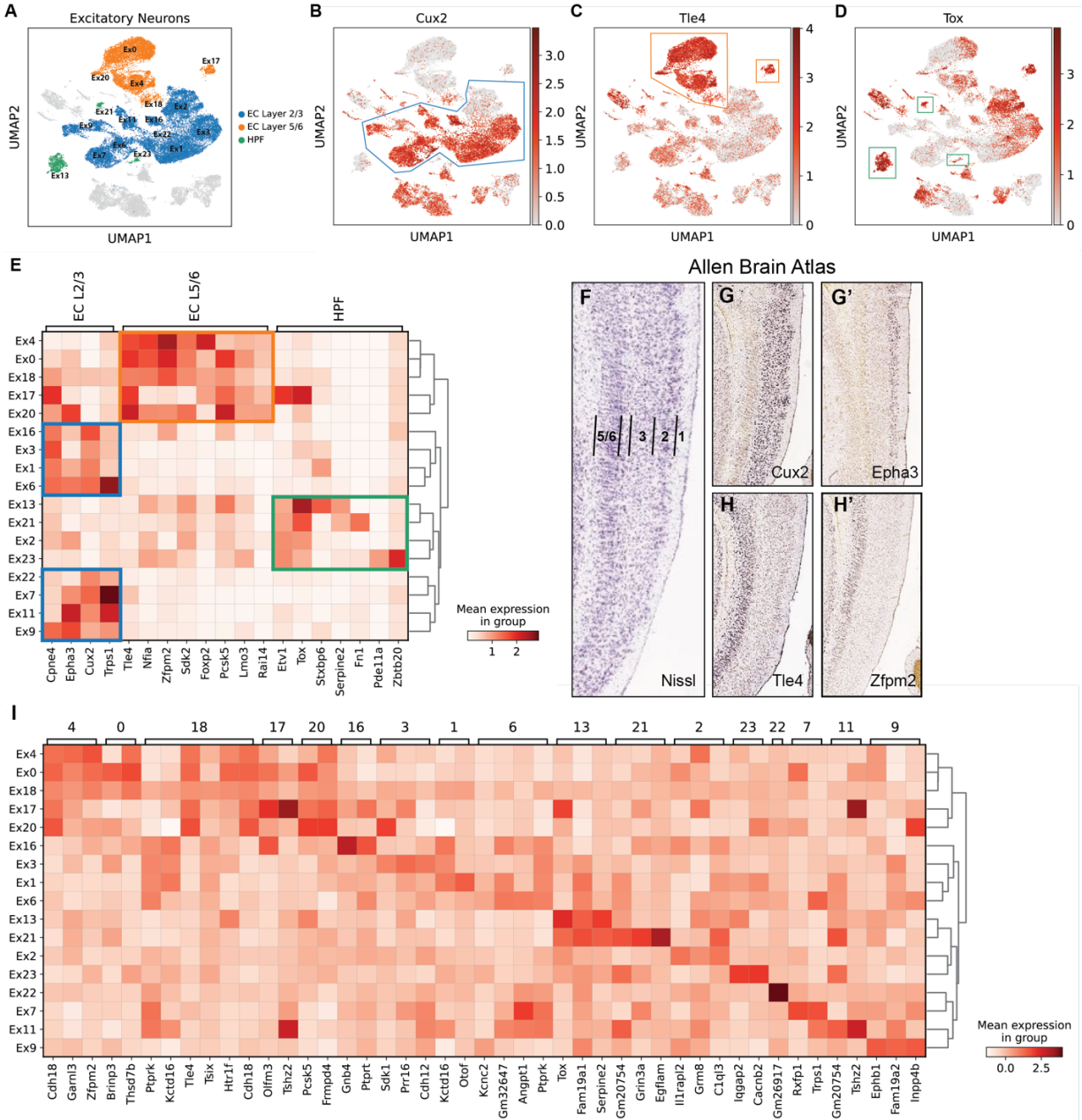
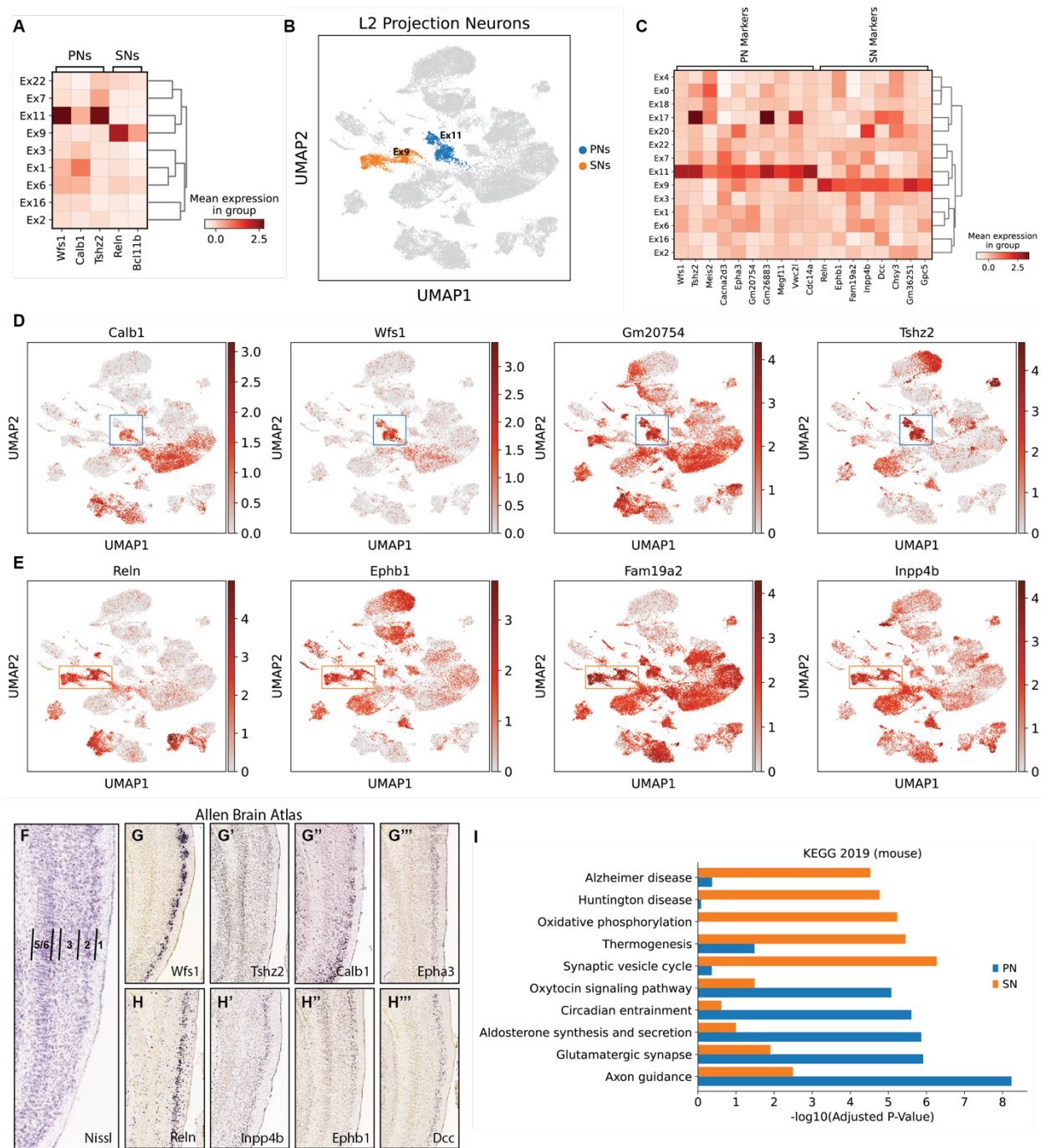


FIGURE 3.4. Glutamatergic neuron types. (A-D) UMAP representation of annotated excitatory neuron classes (A) and marker gene expression for *Cux2* corresponding to superficial L2/3 EC neurons (B), *Tle4* corresponding to deep L5/6 neurons (C), and *Tox* corresponding to neurons of the HPF and SUB (D). (E) Dendrogram of relatedness between excitatory (Ex) clusters with a matrix plot of key marker gene expression for lamina or region of origin. (F-H) Images obtained from the Allen Brain Atlas data portal of the Developing Mouse Brain. (F) For anatomical reference: Nissl staining of all nuclei in a sagittal section of 28 day old (P28) murine brain with layers 1, 2, 3, 5/6 labeled. Note the relative absence of cell bodies in unlabeled L4, or the *lamina dissecans*. (G-H) ISH expression of key marker genes for superficial L2/3 neurons (G-G') and deep L5/6 neurons (H-H') of EC. (I) Matrix plot of expression level of identifying marker genes discovered using NS-Forest (v2.0). Color intensity in matrix plots represents average gene expression per cluster.

FIGURE 3.5. Identification of SN and PN marker genes and comparison of biological pathways. (A) Dendrogram of relatedness between excitatory (Ex) clusters from EC only with a matrix plot of key SN and PN marker gene expression. (B) UMAP representation of cluster Ex9 and Ex11 annotated as SNs and PNs, respectively. (C) Dendrogram of relatedness between all excitatory (Ex) clusters (from EC and HPF) with a matrix plot of all SN and PN binary marker genes identified by NS-Forest (v2.0). Color intensity in matrix plots represents average gene expression per cluster. (D-E) UMAP representation of gene expression patterns for PN (D) and SN (E) marker genes with corresponding PN or SN clusters boxed in blue or orange, respectively. (F-H) Images obtained from ABA data portal of the Developing Mouse Brain. (F) For anatomical reference: Nissl staining of all nuclei in a sagittal section of 28 day old (P28) murine brain with layers 1, 2, 3, 5/6 labeled. Note the relative absence of cell bodies in unlabeled L4, or the *lamina dissecans*. (G-H) ISH expression of key marker genes for PNs (G-G''') and SNs (H-H''') in EC L2. (I) Bar plot showing $-\log_{10}$ of adjusted P-value of the top 5 KEGG 2019 Mouse biological pathways represented in SNs (top; orange) or PNs (bottom; blue) by GSEA for Python.



3.9 TABLES

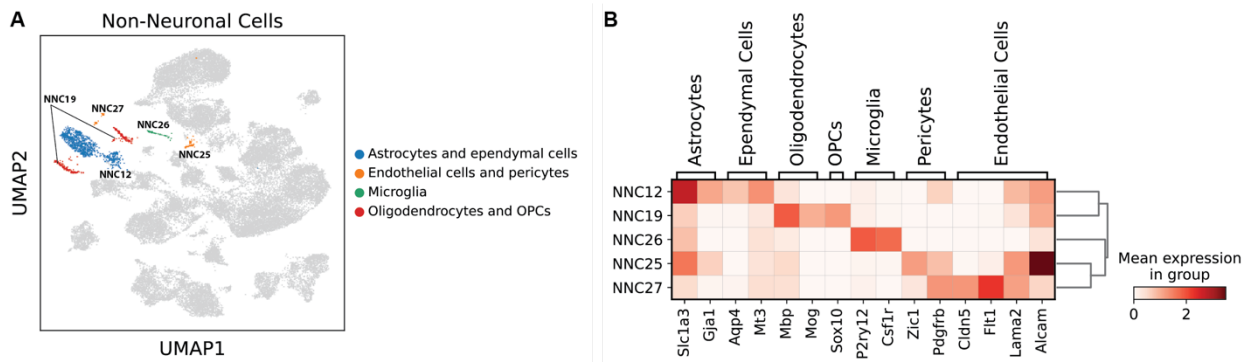
TABLE 3.1. Mouse snRNAseq samples and quality control metrics.

	Sex	Pooled Littermates	Age	Harvest Batch	Sequencing Batch	Nuclei Sequenced	Nuclei Passing QC	Mean Reads/Cell	Mean Genes/Cell
JB_678	Male	4	48d	1	1	9666	9419	49884	3349
JB_826	Mixed	3	48d	1	2	5507	5385	31929	2981
JB_827	Female	3	>1y	2	2	2671	0	36482	2654
JB_828	Male	3	>1y	2	2	12758	12611	30452	2612
JB_829	Female	3	68d	2	2	6206	6091	27025	2132

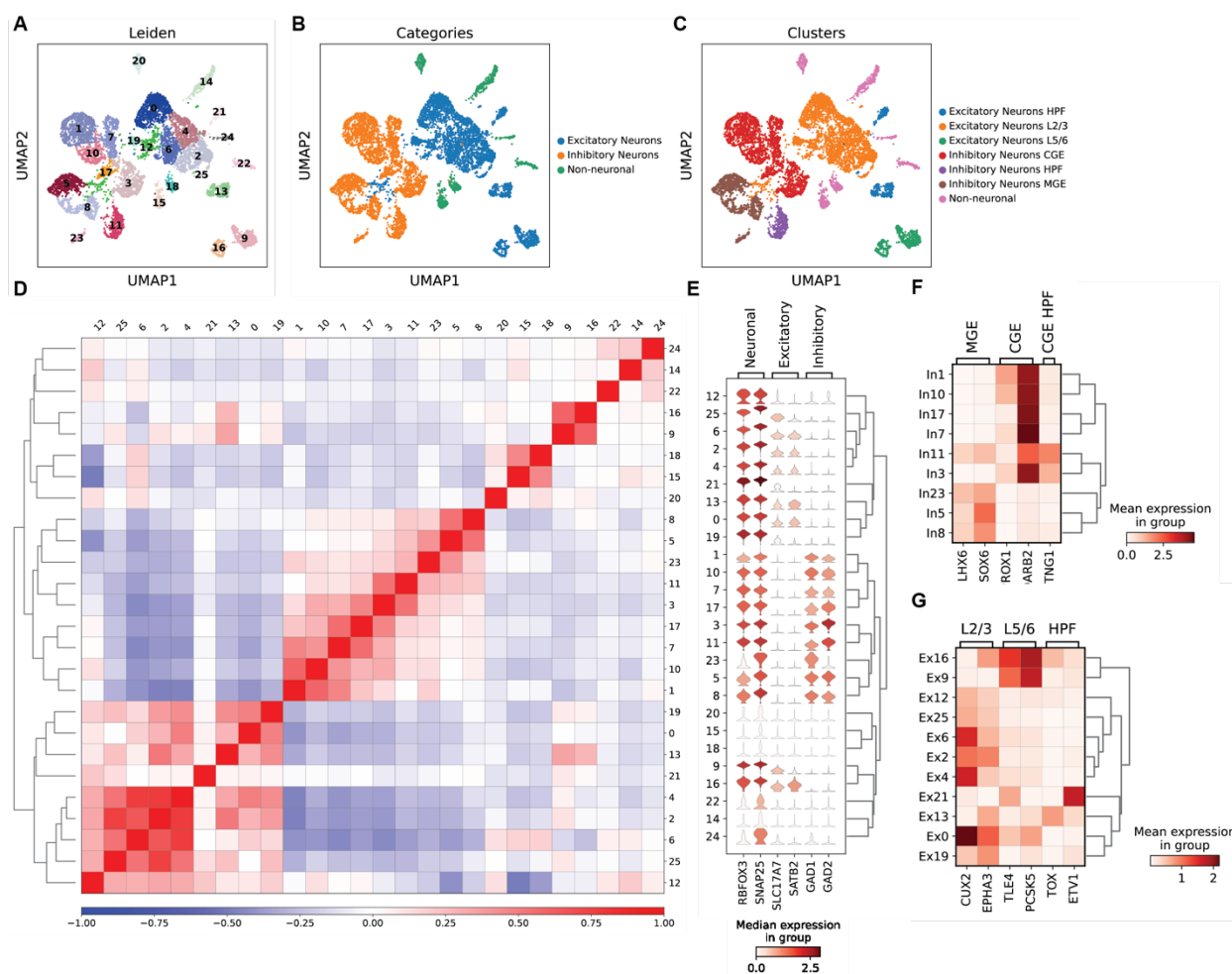
TABLE 3.2. Murine NS-Forest (v2.0) minimal gene markers, region, class, neighborhood, and (if applicable) subclass per cluster at Leiden granularity of 0.6.

Cluster	Annotation	NS-Forest Minimal Marker Genes	Region	Class	Neighborhood	Subclass
0	Ex1_EC_L5/6	Brinp3, Thsd7b	EC	Glutamatergic	L5/6	
1	Ex2_EC_L2/3	Kctd16, Otof	EC	Glutamatergic	L2/3	
2	Ex3_EC_L2/3	Il1rapl2, Grm8, C1ql3	EC	Glutamatergic	L2/3	
3	Ex4_EC_L2/3	Sdk1, Prr16, Cdh12	EC	Glutamatergic	L2/3	
4	Ex5_EC_L5/6	Chd18, Grn13, Zfp102	EC	Glutamatergic	L5/6	
5	In1_EC_Pvalb	Btd11, Kcnc2	EC	GABAergic	MGE	Pvalb
6	Ex6_EC_L2/3	Kcnc2, Gm32647, Angpt1, Ptpkr	EC	Glutamatergic	L2/3	
7	Ex7_EC_L2/3	Rxfp1, Trps1	EC	Glutamatergic	L2/3	
8	In2_EC_Sst	Kcnc2, Grn3a	EC	GABAergic	CGE	Sst
9	Ex8_EC_L2/3_SN	Ephb1, Fam19a2, Inpp4b	EC	Glutamatergic	L2/3	L2 SNs
10	In3_EC_VIP	Synpr, Prox1	EC	GABAergic	CGE	Vip
11	Ex9_EC_L2/3_PC	Gm20754, Tshz2	EC	Glutamatergic	L2/3	L2 PNs
12	NNC1_Ast-Epend	Slc1a2	EC	Non-neuronal	Glia	Astrocytes and ependymal cells
13	Ex10_HPF	Tox, Fam19a1, Serpine2	HPF	Glutamatergic	Other	
14	In4_HPF_Ntng1	Zfmx3	HPF	GABAergic	CGE	Ntng1
15	In5_EC_ReIn/Npy	Fgf13, Adarb2	EC	GABAergic	CGE	ReIn/Npy
16	Ex10_EC_L2/3	Gnb4, Ptptr	EC	Glutamatergic	L2/3	
17	Ex11_EC_L5/6	Olfm2, Tshz2	EC	Glutamatergic	L5/6	
18	Ex12_EC_L5/6	Ptpkr, Kctd16, Tle4, Tsix, Htr1f, Cdh18	EC	Glutamatergic	L5/6	
19	NNC2_Oligo_OPC	Mbp, St18	EC	Non-neuronal	Glia	Oligodendrocytes and OPCs
20	Ex13_EC_L5/6	Pcsk5, Frmpd4	EC	Glutamatergic	L5/6	
21	Ex14_HPF	Gm20754, Grn3a, Egflam	HPF	Glutamatergic	Other	
22	Ex15_EC_L2/3	Gm26917	EC	Glutamatergic	L2/3	
23	Ex16_HPF	Iqgap2, Cacnb2	HPF	Glutamatergic	Other	
24	In6_EC_Pvalb	Trps1, Sdk1	EC	GABAergic	MGE	Pvalb
25	NNC3_Endo-Peri	Ptgds, Cped1	EC	Non-neuronal	Endothelial	Endothelial cells and pericytes
26	NNC4_Micro	Hexb	EC	Non-neuronal	Glia	Microglia
27	NNC5_Endo-Peri	Igf1bp7	EC	Non-neuronal	Endothelial	Endothelial cells and pericytes

3.10 SUPPLEMENTARY INFORMATION



SUPPLEMENTAL FIGURE 3.6. Non-neuronal cell type identification. (A) UMAP representation of annotated NNCs with corresponding cluster number(s). (B) Dendrogram of relatedness between NNC clusters with a matrix plot of key marker gene expression. Color intensity in matrix plots represents average gene expression per cluster.



SUPPLEMENTAL FIGURE 3.7. Preliminary transcriptomic profile of human EC. (A-C) UMAP representation of single nuclei labeled by Leiden clustering and numbering (A), broad classification into non-neuronal cells, excitatory neurons, and inhibitor neurons (B), and more specific subclasses (C). (D) Correlation plot displaying the Pearson correlation coefficient for each cluster as compared to all other clusters as they are ordered by dendrogram. (E) Dendrogram of relatedness between clusters accompanying a stacked violin plot of raw expression data of basic neuronal marker genes. Color intensity represents average expression level of each gene per cluster, while the shape shows the distribution of cells with high or low expression. (F-G) Dendrogram of relatedness between inhibitory (In) (F) and excitatory (Ex) (G) clusters with matrix plots of key marker gene expression. Color intensity in matrix plots represents average gene expression per cluster.

SUPPLEMENTAL TABLE 3.3. Full NS-Forest (v2.0) output

cluster Name	f-measure	marker Count	NSForest_Markers	True Positive	True Negative	False Positive	False Negative	index	Binary_Genes
0	0.840978593	2	['Brinp3', 'Thsd7b']	2585	29023	182	1716	0&Bbrinp3>=1.3531522154808044&Thsd7b>=1.4346174597740173	['Rxfp1', 'Galnt18', 'Brinp3', 'Cdh18', 'Nr4a2', 'Thsd7b', 'Hs3st4', 'Meis2', 'Pcsk5', 'Cobll1']
1	0.670626644	2	['Kctd16', 'Otof']	1479	29334	313	2380	1&Kctd16>=0.7906195521354675&Otof>=1.6720717549324036	['Rgs6', 'Rfx3', 'Kctd16', 'Nectin3', 'Glis3', 'Otof', 'Gpc6', 'Dgkb', 'Fam19a1', 'Prkg1']
2	0.63559322	3	['Il1rapl2', 'Grm8', 'C1ql3']	1125	29963	269	2149	2&Il1rapl2>=0.6551052927970886&Grm8>=0.03169289603829384&C1ql3>=1.1707623600959778	['Cnr1', 'Stgalmac5', 'Hs3st2', 'Il1rapl2', 'Grm8', 'C1ql3', 'Dgkb', 'Kcnt2', 'Ncam2', 'Etv1']
3	0.554160126	3	['Sdk1', 'Prr16', 'Cdh12']	706	30542	194	2064	3&Sdk1>=0.7801139652729034&Prr16>=1.0812230110168457&Cdh12>=0.9462795555591583	['Lhfp', 'Sdk1', 'Rcan2', 'Zmat4', 'Prr16', 'Cdh12', 'Thsd7a', 'Kcnh5', 'Rorb', 'Col26a1']
4	0.563728375	3	['Cdh18', 'Garnl3', 'Zfp6m2']	958	30482	547	1519	4&Cdh18>=0.7025928795337677&Garnl3>=1.3567059636116028&Zfp6m2>=1.3776822686195374	['Thsd7b', 'Cdh18', 'Garnl3', 'Zfp6m2', 'Col5a1', 'Hs3st4', 'Fmpod4', 'Grm8', 'Foxp2', 'Mgat4c']
5	0.792027954	2	['Btd11', 'Kcnc2']	1224	31317	214	751	5&Btd11>=2.1583141088485718&Kcnc2>=1.9519274234771729	['Slit2', 'Dock4', 'Btd11', 'Kcnc2', 'Nek7', 'Gabrg3', 'Kcnmb2', 'Sox6', 'Erbb4', 'Nxph1']
6	0.511022044	4	['Kcnc2', 'Gm32647', 'Angpt1', 'Ptprk']	816	30856	690	1144	6&Kcnc2>=0.6529567539691925&Gm32647>=0.2490515410900116&Angpt1>=0.13752441853284836&Ptprk>=0.16477081179618835	['Kcnc2', 'Rfx3', 'Dpyd', 'Gm32647', 'Angpt1', 'Ptprk', 'Ptprt', 'Inpp4b', 'Trps1', 'Fign']
7	0.836277174	2	['Rxfp1', 'Trps1']	1231	31463	131	681	7&Rxfp1>=1.519375741481781&Trps1>=1.1462329030036926	['Chrm3', 'Rxfp1', 'Trps1', 'Ntng1', 'Nell1', 'Reig', 'Cemip', 'Plch1', 'Gm6602', 'Fermt1']
8	0.773854245	2	['Kcnc2', 'Grin3a']	824	31739	87	856	8&Kcnc2>=1.2002124786376953&Grin3a>=2.316055178642273	['Trhde', 'Rab3b', 'Kcnc2', 'Synpr', 'Grin3a', 'Grik1', 'Sst', 'Grip1', 'Kcnmb2', 'Nxph1']
9	0.742062515	3	['Ephb1', 'Fam19a2', 'Inpp4b']	603	32092	79	732	9&Ephb1>=1.0799004435539246&Fam19a2>=1.202382504940033&Inpp4b>=1.1607545614242554	['Ephb1', 'Fam19a2', 'Chsy3', 'Gpc5', 'Gm36251', 'Inpp4b', 'Reln', 'Dcc']
10	0.729626361	2	['Synpr', 'Prox1']	496	32187	32	791	10&Synpr>=2.051627039909363&Prox1>=2.5354602336883545	['Cnr1', 'Slc44a5', 'Synpr', 'Sorcs3', 'Npas3', 'Prox1', 'Grip1', 'Vip', 'Adarb2', 'Dlx6os1']
11	0.84996478	2	['Gm20754', 'Tshz2']	724	32368	75	339	11&Gm20754>=1.1867761611938477&Tshz2>=2.1282634735510742	['Meis2', 'Gm20754', 'Epha3', 'Megf11', 'Gm26883', 'Tshz2', 'Wfs1', 'Vwcz1', 'Cdc14a', 'Cacna2d3']

SUPPLEMENTAL TABLE 3.3. Full NS-Forest (v2.0) output, continued

cluster Name	f-measure	marker Count	NSForest_Markers	True Positive	True Negative	False Positive	False Negative	index	Binary_Genes
12	0.921052632	1	['Sic1a2']	686	32580	18	222	12&Sic1a2>=2.1574206352233887	['Wdr17' 'Sic1a2' 'Sparcl1' 'Prex2' 'Cst3' 'Gpc5' 'Plpp3' 'Apoe' 'Sic1a3' 'Mertk']
13	0.72425829	3	['Tox', 'Fam19a1', 'Serpine2']	332	32653	37	484	13&Tox>=2.0346851348876953&Fam19a1>=1.256161391735077&Serpine2>=1.25694078207016	['Stxbp6' 'Pbx3' 'Tox' 'Ctnn6' 'Fam19a1' 'Serpine2' 'L3mbtl4' 'Sorcs1' 'Tenn3' 'Gm2164']
14	0.499325236	1	['Zfhx3']	296	32527	267	416	14&Zfhx3>=3.793079137802124	['Rmst' 'Usp29' 'Pbx3' 'Htr2c' 'Zfhx3']
15	0.84824501	2	['Fgf13', 'Adarb2']	493	32740	56	217	15&Fgf13>=2.7897614240646362&Adarb2>=1.8517467379570007	['Nyap2' 'Kit' 'Fgf13' 'Crispld1' 'Adarb2' 'Sv2c' 'Gad2' 'Alk' 'Egfr' 'Reln']
16	0.570107858	2	['Gnb4', 'Ptprt']	148	32917	39	402	16&Gnb4>=3.531391143798828&Ptprt>=1.6836904287338257	['Sh3gl3' 'Sh3d19' 'Rgs12' 'Adamts1' 'Gnb4' 'Ptprt' 'Cadps2' 'Ntng2' 'Oprk1' 'Tmem163']
17	0.817825661	2	['Olfm3', 'Tshz2']	167	33159	2	178	17&Olfm3>=2.0362404584884644&Tshz2>=3.3152533769607544	['Rgs6' 'Olfm3' 'Stard5' 'Gm26883' 'Tshz2' 'Prkca' 'Etv1' 'Lcp1' 'Vwc2' 'Mgat4c']
18	0.396551724	6	['Ptprk', 'Kctd16', 'Tle4', 'Tsix', 'Htr1f', 'Cdh18']	138	32957	213	198	18&Ptprk>=0.6229796409606934&Kctd16>=0.6229796409606934&Kctd16>=0.3584800958633423&Tle4>=0.22047040611505508&Tsix>=0.6658549308776855&Htr1f>=0.08554381504654884&Cdh18>=0.3793899863958359	['Ptprk' 'Kctd16' 'Tle4' 'Tsix' 'Htr1f' 'Cdh18' 'Zfpm2' 'Thsd7b' 'Hs3st4' 'Xist']
19	0.746971736	2	['Mbp', 'St18']	111	33207	0	188	19&Mbp>=4.61466908454895&St18>=7.391859292984009	['Tnr' 'Mbp' 'Pde4b' 'Lhfp13' 'St18' 'Ugt8a' 'Plp1' 'Bcas1' 'Sox10' 'Gjc3']
20	0.658567775	2	['Pcsk5', 'Frmpd4']	103	33199	21	183	20&Pcsk5>=2.1210345029830933&Frmpd4>=1.9416038990020752	['Tmem132d' 'Pcsk5' 'AC129186.1' 'Frmpd4' 'Sdk1' 'Fbx17' 'Lhfp13' 'Kcnab1' 'Inpp4b' 'Ctgf']
21	0.61971831	3	['Gm20754', 'Grin3a', 'Egflam']	44	33351	8	103	21&Gm20754>=1.3943430185317993&Grin3a>=1.8738834857940674&Egflam>=3.911966323852539	['Pdzd2' 'Tmtc2' 'Gm20754' 'Grin3a' 'Fam19a1' 'Egflam' 'Sorcs1' 'Otx1' 'Reln' 'Fn1']
22	0.693512304	1	['Gm26917']	62	33349	14	81	22&Gm26917>=5.043304443359375	['Lars2' 'Gm26917' 'Peak1' 'Taco1' 'Hs6st3' 'Cmss1']
23	0.597014925	2	['lqgap2', 'Cacnb2']	32	33372	2	100	23&lqgap2>=2.860042095184326&Cacnb2>=2.3336821794509888	['Gm13269' 'lqgap2' 'Gm10848' 'Gm10754' 'Ndstd4' 'Cacnb2' 'Gm2115' 'Pde11a' 'Gm2164' 'Zbtb20']

SUPPLEMENTAL TABLE 3.3. Full NS-Forest (v2.0) output, continued

cluster Name	f-measure	marker Count	NSForest_Markers	True Positive	True Negative	False Positive	False Negative	index	Binary_Genes
24	0.821596244	2	['Trps1', 'Sdk1']	35	33433	0	38	248&Trps1>=2.0649536848068237&Sdk1>=2.536691904067993	['Trps1' 'Nyap2' 'Thsd7a' 'Sdk1' 'Zfp804b' 'Cntnap5b' 'Ano4' 'Inpp4b' 'Unc5b' 'Vmn1r209']
25	0.801104972	2	['Ptgds', 'Cped1']	29	33444	1	32	258&Ptgds>=9.207725524902344&Cped1>=9.9968581199646	['Bicc1' 'Trpm3' 'Alcam' 'Ptgds' 'Ranbp3l' 'Cped1' 'Sic6a20a' 'Tbx15' 'Zic1' 'Sic6a13']
26	0.85492228	1	['Hexb']	33	33445	0	28	268&Hexb>=7.013556718826294	['Pde3b' 'P2ry12' 'Plxdc2' 'Cx3cr1' 'Hexb' 'Tgfb1r' 'Apbb1ip' 'Csf1r' 'Arhgap45' 'Siglech']
27	0.720338983	1	['lgfbp7']	17	33468	4	17	278&lgfbp7>=9.061253070831299	['Bsg' 'Dlc1' 'Ets1' 'lgfbp7' 'Lef1' 'Atp13a5' 'Ebf1' 'Ftl1']

Chapter 3, in part, is currently being prepared for submission for publication of the material: Koehler, Alanna E.; Zhang, Yun; Tedeschi, Neil; Daza, Ray Anthony M.; Scheuermann, Richard H.; Hevner, Robert F. Transcriptomic analysis of neuronal subtypes in entorhinal cortex. In preparation. The dissertation author was the primary investigator and author of the material contained in this chapter and is co-primary investigator and author of the corresponding manuscript submitted for publication.

CHAPTER 4:

Discussion of the Dissertation

4.1 CONCLUSIONS

The overarching goal of this dissertation was to better understand the heterogeneity of neurons within EC, an under-studied brain region with a non-canonical neurogenic gradient and selective vulnerability to devastating neurodegenerative disease. Chapter 1 provided an overview of the state of the field, including primers on cerebral cortex anatomy, corticogenesis, and the organization and function of EC. We discussed seminal work exploring functional and molecular EC neuron types, birthdating of EC cells, and utilizing snRNAseq to profile EC neurons and address neuronal selective vulnerability to AD.

In Chapter 2, we hypothesized that some EC neurons diverge from classic “inside-out” neurogenesis, exhibiting a distinct differentiation pattern. We investigated the developmental timelines of EC L2 principal neurons, including Reelin⁺ SNs, which peculiarly express deep-layer neocortical markers. We discovered an early-born population of Reelin⁺ SNs, which are produced throughout the span of EC neurogenesis (E10.5-E15.5). In contrast, Calbindin⁺ PNs did not exhibit substantial neurogenesis until E13.5-E15.5, which is more typical of upper-layer neurons. Despite differing neurogenic timelines, at least a subset of both Reelin⁺ SNs and Calbindin⁺ PNs were derived from Tbr2⁺ IPCs rather than directly from NECs or RGCs. We therefore conclude that,

although EC Reelin⁺ SNs are a unique upper-layer neurons with precocious development and gene expression patterns typical of deep-layer neurons, they are not the result of genesis from a different progenitor type than neighboring, later-born PN. This is the first study, to our knowledge, to examine EC neuron subtype genesis as early as E10.5 and to lineage trace SNs and PNs to IPCs.

In Chapter 3, we begin to compile two snRNAseq atlases of neuronally-enriched EC nuclei: one consisting of >30,000 murine neurons and another very preliminary atlas with >7,000 human neurons. In addition to identifying canonical GABAergic interneuron subtypes (which vary little between cortical regions (Tasic et al., 2016; Yao et al., 2021)), we annotate lamina-specific EC glutamatergic neurons, providing necessary and sufficient minimal marker genes for each neuronal cluster in mice. We also report a list of binary marker genes for SNs and PNs, several of which are novel markers of these cell types not yet found in the literature. Finally, we begin to examine biological pathway enrichment for murine SNs and PNs towards our goal of gaining better functional understanding of these cell types. While much of this work is preliminary, we successfully identify PNs and SNs in mice, present novel marker genes for their identification and classification, and suggest differentially enriched biological pathways between the two that may make SNs more susceptible to disease.

4.2 FURTHER ELUCIDATING EC NEURON DEVELOPMENT

One potential method for further interrogating EC neuron development would be to use clonal analysis using the mosaic analysis with double markers (MADM) (Zong et al., 2005). Our lab previously utilized our IPC-specific Eomes^{CreERT2} mouse model (Pimeisl et al., 2013) crossed with Madm11 transgenic mice (Hippenmeyer et al., 2010) to sparsely and permanently label early IPCs and their progeny were with either TdTomato red fluorophore or enhanced green fluorescent

protein (eGFP) in a tamoxifen-dependent manner (Mihalas and Hevner, 2018). This allowed us to trace a small cohort of early IPC-derived neurons that were born late in corticogenesis and migrated to upper cortical layers, indicating that some early IPCs either (1) did not immediately differentiate but rather continued proliferating or remained quiescent until late neurogenesis or (2) divided asymmetrically to produce both lower- and upper-layer neurons (Mihalas et al., 2016). It would be interesting to combine this method with IHC in the EC to detect clone-specific development of SNs. This would address whether precocious SNs derive from early IPCs capable of producing both upper- and lower-layer neurons.

3.4 DEVELOPING THE ULTIMATE RESOURCE FOR EC NEURONS

A future goal is to further develop robust, meticulously-annotated atlases of both murine and human EC neurons, including sampling more nuclei for better granularity and applying insightful transcriptome-based taxonomy in order to compile an EC cell type ontology. To this end, we are partnering with Richard Scheuermann's Lab at the J. Craig Venter Institute to expand our analyses and generate semantic cell type definitions for EC neurons. His laboratory has been fundamental in developing tools for better single cell data exploration, including NS-Forest (v2.0), used here to identify minimal marker genes and lists of genes with high binary expression for each cell cluster. When combined with functional and laminar gene expression profiles and, when available, morphological, spatial, and/or electrophysiological data, these minimal marker genes can be used to generate a naming schema that informs function and allows for easier cross-identification of similar neuronal populations in other datasets (Aevermann et al., 2021). This data can be shared to a large ontology focused on specific cell type profiles (Osumi-Sutherland et al., 2021), the likes of which are already being developed, including the Provisional Cell Ontology

(PCL) on BioPortal (Whetzel et al., 2011) by the National Center for Biomedical Ontogeny (NCBO) (<https://bioportal.bioontology.org/ontologies/PCL>) and The Cell Ontology (CL) by The Open Biological and Biomedical Ontology (OBO) Foundry (Diehl et al., 2016).

Such ontologies are even better informed with spatial transcriptomics data, which provides more specific information about the location of a specific cell type within a section of tissue or, in some cases, even information of the subcellular localization of specific marker gene transcripts within single cells. Platforms enabling this type of analysis include Visium by 10x Genomics, GeoMx by Nanostring, and various types of fluorescent ISH (FISH), including multiplexed error-robust fluorescence in situ hybridization (MERFISH) (Chen et al., 2015). In-depth explorations of EC neuron types by these methodologies would add an even greater layer of specificity and complexity to a cell type ontology.

Another useful technology worth mentioning is Patch-Seq (Cadwell et al., 2015), which allows for combinational profiling of electrophysiology, morphology, and transcriptome of individual neurons. However, this method is low-throughput, labor intensive, and requires a broad range of highly specialized technical and analytical skills to master. It is also not compatible with droplet-based snRNAseq (Lipovsek et al., 2021).

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