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Investigating Roles of Store-Operated Calcium Entry in the Developing Cerebral Cortex

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Investigating Roles of Store-Operated Calcium Entry in the Developing Cerebral Cortex

by

Arpana Arjun McKinney

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Dedicated to Moham Rangaswamy and Radha Natarajan

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GP and **AAM** designed the study, wrote the paper and performed the experiments. VP, JT, SL, YK, and CH performed experiments and provided technical assistance. GP and ARK provided expertise and resources.

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Investigating Roles of Store-Operated Calcium Entry in the Developing Cerebral Cortex

Arpana Arjun McKinney

Abstract

Calcium waves, induced by extracellular agonists and sustained by intraorganellar calcium stores, have been suggested to influence the proliferation of neural stem and progenitor cells (NSPCs) of the developing cerebral cortex. It remains unclear, however, how NSPC calcium stores are regulated and how calcium dynamics are transduced into NSPC behaviors. This thesis work begins to tackle this central question by investigating how store operated calcium entry (SOCE), a calcium influx pathway tied to emptying of endoplasmic reticulum (ER) calcium stores, regulates calcium signaling in cortical NSPCs to influence their behavior and output. To this end, we used calcium imaging to successfully record SOCE in mouse and human cortical NSPCs. By isolating enriched populations of embryonic cortical cells using an *in vivo* labeling technique called "FlashTag," we also found that *Orai1*, *Orai2* and *Stim2* encode the primary mediators of SOCE during development. Moreover, their expression is dynamically regulated during NSPC lineage progression. In line with this, conditional deletion of *Orai1* and *Orai2* in the excitatory lineage eliminates SOCE in embryonic NSPCs. Orthogonal pharmacological experiments revealed that SOCE is required in both mouse and human primary cortical NSPCs to maintain proliferation, and robust SOCE responses in proliferative NSPCs are significantly diminished upon cell cycle exit.

Supporting this idea, *in utero* electroporation of STIM2 isoforms with opposing effects on SOCE bidirectionally regulates cell cycle exit of cortical NSPCs.

In an effort to define upstream regulators of SOCE in the developing cortex, we found that activation of P2Y1 receptor-mediated purinergic signaling induces SOCE. Importantly, SOCE is abrogated upon purinergic receptor activation in NSPCs lacking *Orai1* and *Orai2*, suggesting that SOCE may transduce purinergic signals in the developing cortex to modulate proliferation. Interestingly, our preliminary analyses suggest that dual conditional deletion of *Orai1/2* does not grossly alter NSPC proliferation across the entire NSPC compartment in the embryonic cortex. This suggests the possibility that proliferation may be regulated at a cell type-specific level in the context of conditional *Orai1/2* inactivation, an idea that has been suggested in the context of other modulators of NSPC proliferation. Collectively, our data suggest that dynamic regulation of SOCE mediators and downstream calcium signaling plays indispensable roles in the control of stem/progenitor cell biology in the developing cortex. As aberrant calcium signaling has been implicated in neurodevelopmental disorders, our studies defining the molecules and mechanisms involved in transducing calcium signals during cortical development will provide essential insights into normal and dysfunctional corticogenesis.

Chapter 1 and 2 of this thesis provide an introduction to calcium signaling in the developing cortex, with an emphasis on roles of aberrant calcium regulation and electrical activity in the etiology of neurodevelopmental disease. Through a detailed discussion of the known mechanisms by which calcium directs cellular behaviors in the developing cortex, we posit that understanding the normal events that build the nervous system relies on gaining insight into cell type-specific calcium signaling mechanisms and that these mechanisms may be disrupted or reactivated in the

context of neurological disease. Chapter 3 focuses on the regulation of SOCE during embryonic cortical development. Finally, Chapter 4 concludes this thesis with a discussion of the intersection of different modes of calcium entry, the reactivation of developmental calcium signaling mechanisms in disease states, and a brief overview of ongoing work that we have been pursuing aimed at defining cell type specific regulators of calcium signaling using long isoform sequencing.

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Chapter 1 – Calcium and activity-dependent signaling in the developing cerebral cortex

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1.1 – Introduction

Calcium, which lies at the hub of multiple signal transduction pathways, is uniquely situated to transduce dynamic biological inputs into distinct cell behaviors (Berridge et al., 2003). Calcium influx in the embryonic brain occurs in response to multiple developmental signals, including electrical activity, and dynamic elevations in cytoplasmic calcium are linked to transcriptional programs critical for development, homeostasis and plasticity (Greer & Greenberg, 2008; Lyons & West, 2011). In the introductory chapters of this thesis, we present evidence supporting the idea that spatiotemporally regulated and cell-specific functions of calcium signaling transducers underlie the earliest cellular behaviors that build the cerebral cortex. We focus on calcium entry in developing cortical cells, incorporating lessons gleaned from other developing neural populations. We also detail cell biological outputs influenced by precise control of cytoplasmic calcium, placing

special emphasis on stem/progenitor populations and immature neuroblasts. Moreover, we discuss genetic evidence suggesting that deregulation of intracellular calcium represents a potential node of convergence for neurodevelopmental disorders.

1.2 – Ion channels and pumps mediating calcium homeostasis

In resting neural cells, low cytosolic calcium is maintained by plasma membrane calcium ATPases (PMCA1-4), which extrude calcium from cells, and pumps on the surface of the endoplasmic reticulum (ER) and mitochondria (e.g., the ER calcium ATPases SERCA1-3), which transport calcium into intraorganellar stores (Clapham, 2007). Calcium equilibria can be transiently disrupted by calcium influx from the extracellular space through calcium-permeable channels (**Figure 1.1**) (Clapham, 2007). Large calcium elevations are countered by sodium calcium exchangers (NCX1-4) on the plasma membrane and mitochondrial and ER membranes (Clapham, 2007). Here we focus on channels mediating two types of calcium entry in immature neural cells that are dependent on environmental signals and result in downstream calcium-dependent signaling and transcription (**Figure 1.1**).

1.2.1 Voltage-gated calcium channels

Calcium entry through voltage-gated calcium channels (VGCCs) is a primary mechanism of calcium influx in the developing nervous system. By converting changes in membrane potential at the cell surface into intracellular calcium elevations, VGCCs couple electrical activity to downstream cell biological processes, including phosphorylation and transcription (Catterall, 2011; Zamponi et al., 2015). These channels are composed of a pore-forming α subunit, which allows calcium entry in response to depolarizing stimuli, and auxiliary β, α2δ, and γ subunits that

regulate channel surface expression and functional properties (Catterall, 2011; Zamponi et al., 2015). VGCCs can be divided into broad families based on their properties: L-type channels of the Ca_v1 family; P-, Q-, N-, and R- type channels of the Ca_v2 family; and T-type channels of the Ca_v3 family (Catterall, 2000, 2011; Ertel et al., 2000; Zamponi et al., 2015). Ca_v1 and Ca_v2 channels are activated at high voltages, whereas T-type channels are low voltage-activated. Within these classes, specific family members display unique properties, as well as distinct subcellular and tissue-specific localization, resulting in channels that play distinct roles in different tissues and unique combinations of deficits associated with mutations in specific VGCCs (Catterall, 2011; Zamponi et al., 2015).

1.2.2 Store operated calcium entry

A second major source of calcium influx in the developing brain is store-operated calcium entry (SOCE), which is activated upon depletion of ER calcium stores in response to extracellular signals (R. S. Lewis, 2007; Prakriya & Lewis, 2015). The key players in SOCE are the ORAI plasma membrane channels, ORAI1-3 (also referred to as Calcium Release-Activated Calcium, or CRAC, channels), and the Stromal Interaction Molecule (STIM) family of ER calcium sensors, STIM1 and STIM2. Activation of plasma membrane receptor tyrosine kinases or G-protein coupled receptors by extracellular ligands (e.g. growth factors) promotes inositol trisphosphate (IP3)- or ryanodine-mediated release of calcium into the cytosol from the ER lumen (R. S. Lewis, 2007; Prakriya & Lewis, 2015). When intraluminal calcium is depleted, STIM proteins on the ER membrane oligomerize and translocate to ER-plasma membrane junctions, trapping and interacting with plasma membrane ORAI channels to allow calcium influx (R. S. Lewis, 2007). STIM1 potently activates SOCE through its interactions with ORAI channels (C. Y. Park et al.,

2009). Calcium entry mediated by STIM2 plays an important role in homeostatic regulation of basal cytoplasmic levels (Brandman et al., 2007), though its precise function remains debated (see Chapter 3). In physiological conditions, SOCE elicits a slow influx of calcium through ORAI channels that can last on the timescale of minutes to hours. Importantly, SOCE can generate distinct patterns of intracellular calcium fluctuations, including oscillatory signals, to precisely regulate various molecular events, including transcriptional activation, and different cellular behaviors (Dolmetsch & Lewis, 1994; R. S. Lewis, 2011; Somasundaram et al., 2014).

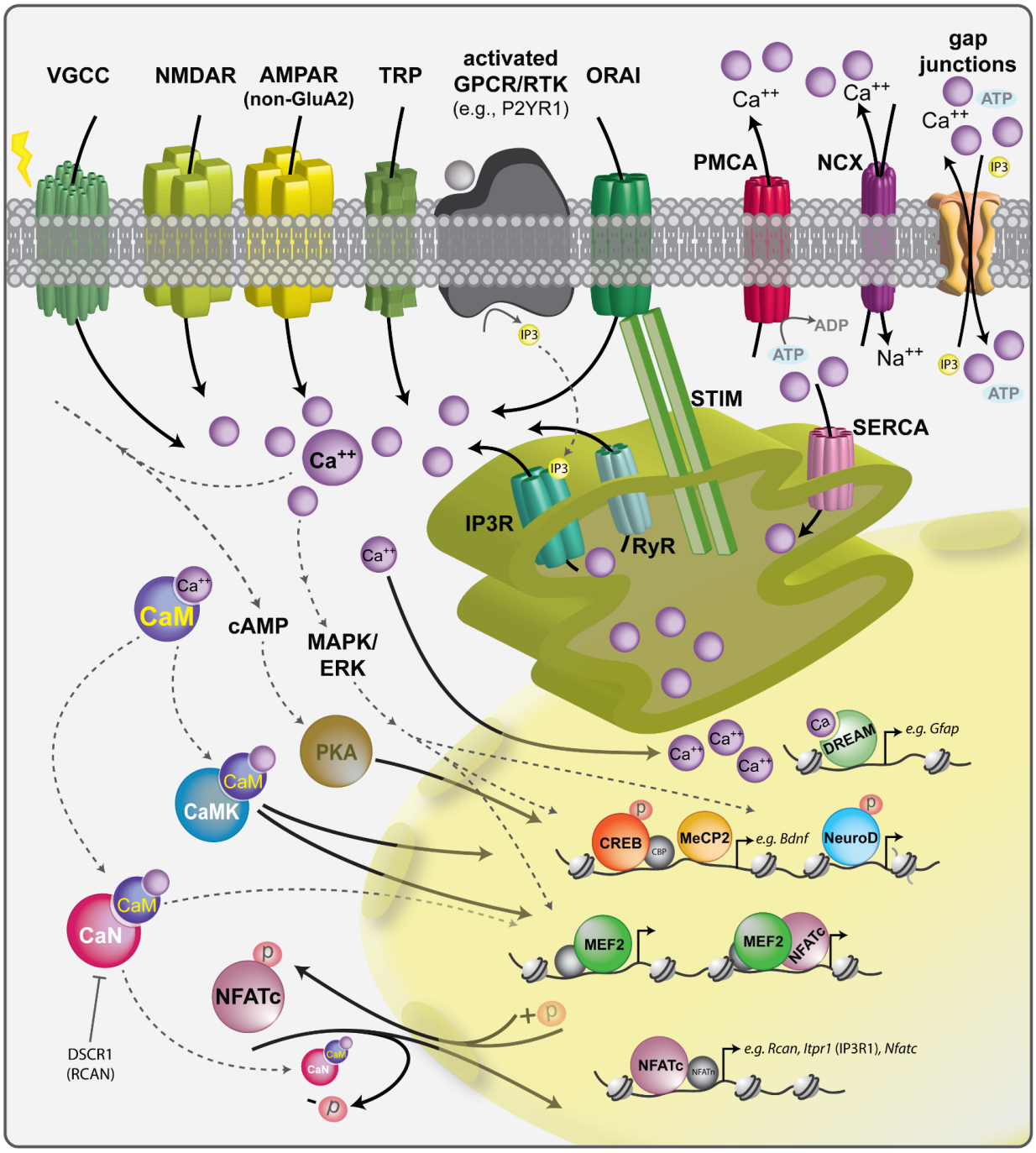

Figure 1.1 – Overview of intracellular calcium signaling
Schematic highlighting ion channels and pumps mediating cytoplasmic calcium homeostasis and select calcium-dependent pathways that transduce calcium signals to the nucleus. Low resting calcium levels are maintained by plasma membrane calcium ATPases (PMCA1-4, encoded by *ATP2B1-4*), which actively extrude calcium out of the cell and display low capacity for calcium efflux but high calcium affinity, and sarco/endoplasmic reticulum (SR/ER) calcium ATPases (SERCA1-3, encoded by *ATP2A1-3*), which transport calcium from the cytoplasm into the ER.

Calcium-permeable channels mediating calcium influx from the extracellular space include voltage-gated calcium channels (VGCCs), N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs), transient receptor potential (TRP) channels and ORAI channels. VGCCs are activated by membrane depolarization, whereas ORAI channels allow calcium influx upon ER calcium store depletion. Successive release of calcium from the ER, mediated by IP3R or RyR, depletes ER calcium, which in turn activates STIM calcium sensors to promote their interaction with ORAI channels and subsequent calcium influx. Gap junctions in the plasma membrane allow for intercellular propagation of calcium signals, through direct transfer of ions or small molecules critical for intracellular signaling (e.g., IP3). Different upstream calcium signals regulate specific calcium-dependent signaling pathways and gene expression programs. Calcium-sensitive proteins in the cytoplasm (e.g., Calmodulin, CaM) undergo a conformational change upon calcium binding, initiating distinct signaling cascades to the nucleus that culminate in calcium-dependent transcription. Select calcium-dependent transcription factors with reported roles in the developing nervous system are highlighted here. Other organellar calcium stores (e.g., mitochondria, lysosomes, Golgi apparatus) are not depicted.

1.3 - Mechanisms regulating intracellular calcium signaling

Calcium signaling involves both the sensing and transduction of extracellular calcium (Brown et al., 1993), as well as the transport of calcium across the plasma membrane. Homeostatic tuning of intracellular calcium employs an extensive repertoire of cell surface and organellar ion channels, transporters, pumps and buffers (**Figure 1.1**), each possessing distinctive properties (e.g., selectivity, conductance, calcium affinity, kinetics). These effectively stage a dynamic process of intracellular compartmentalization, wherein calcium abundance in different subcellular compartments (e.g., cytoplasm, intraorganellar calcium stores) changes across space and time. Movement of calcium into and out of these compartments enables activation of distinct sets of calcium-sensitive proteins, based on their levels and localization (Berridge et al., 2003; Clapham, 2007). How distributed networks of calcium signaling proteins confer signaling specificity in various cellular contexts remains a fundamental question. Below, we briefly describe mechanisms of calcium regulation in developing neural cells. We emphasize spatiotemporal properties of calcium signals, alternative splicing of genes regulating calcium signaling, and cell type-specific

expression of calcium-dependent effectors. Coupled with the cellular niche and environmental signals to which a cell is exposed, the intersection of these factors influences calcium dynamics, ultimately dictating the selective activation of gene expression programs (Clapham, 2007; Rosenberg & Spitzer, 2011).

1.3.1 Spatiotemporal regulation of calcium signals

Calcium influx through different calcium-permeable channels engages distinct signaling cascades (**Figure 1.1**), in part based on the localization of calcium-sensitive signaling proteins (Bading et al., 1993; Graef et al., 1999; Parekh, 2008; West et al., 2001). Cytoplasmic calcium chelators, as well as ion pumps and exchangers that bind or extrude calcium, create transient, spatially-restricted, high-calcium microenvironments within cells (Parekh, 2008). This enables distinct contributions of local and global calcium elevations to intracellular signaling. Functional knock-in experiments and the use of calcium chelators with different affinities have revealed, for example, that local calcium elevations through L-type voltage-gated calcium channels (VGCCs) are transduced to the nucleus via a shuttle protein to promote activation of the transcription factor CREB (Deisseroth et al., 1996; Dolmetsch et al., 2001; H. Ma et al., 2014). In hippocampal neurons, it has been suggested that the calcium/calmodulin-activated phosphatase, calcineurin (CaN), is tethered to L-type VGCCs via an anchoring protein to contribute to the regulation of calcium influx through these channels (Oliveria et al., 2007, 2012).

Local translation of calcium signaling components can also influence calcium dynamics in specific cellular compartments, such as neuronal dendrites (Holt et al., 2019; Sun et al., 2021). Moreover, emerging data reveal that calcium elevations near different intracellular calcium stores (e.g., lysosomes) regulate processes like autophagy (Medina et al., 2015). In dendrites,

endoplasmic reticulum (ER) stores participate in localized calcium signaling critical for synaptic plasticity (Hirabayashi et al., 2017; O'Hare et al., 2022; Takechi et al., 1998). Mitochondrial calcium uptake, regulated by distinct mitochondrial morphologies in different neuronal compartments, can modulate cytosolic calcium levels and processes like neurotransmission (T. L. Lewis et al., 2018). Notably, spatial regulation of calcium is not restricted to individual cells, as propagation of calcium signals can occur across cohorts of coupled cells in the developing brain (Weissman et al., 2004).

Dynamic patterns of calcium fluctuations also enable signaling specificity, encoding information that is translated into long-lasting biochemical changes by cytosolic proteins with different calcium sensitivities (Rosenberg & Spitzer, 2011). In T cells, for instance, different patterns of calcium oscillations control transcriptional specificity (Dolmetsch et al., 1997, 1998). Seminal studies of the developing *Xenopus* nervous system identified two types of spontaneous calcium transients contributing to distinct aspects of spinal neuron differentiation (Gomez & Spitzer, 1999; Gu et al., 1994; Gu & Spitzer, 1995). Recently, temporal waves of gene expression were identified in cortical neurons, resulting from activity patterns of different durations (Tyssowski et al., 2018). The initial wave of early-response transcription factors induced by prolonged depolarization, including NPAS4 (Y. Lin et al., 2008), corresponds to activity-dependent programs activated by brief stimulation. Other studies implicate Nuclear Factor of Activated T-cells (NFAT) transcription factors, previously linked to calcium influx through L-type VGCCs (Graef et al., 1999), as somatic calcium spike counters transducing dendritic VGCC activation to the nucleus (Wild et al., 2019). Dissecting how cell type-specific calcium transients encode information in the embryonic cortex will inform how these dynamics drive cell biological changes to impact developmental programs.

1.3.2 Coordination of routes of calcium entry

VGCCs and store-operated calcium entry (SOCE) are two major sources of calcium influx in developing neural cells (Toth et al., 2016). Growing evidence indicates that these parallel entry mechanisms are reciprocally regulated (C. Y. Park et al., 2010; Y. Wang et al., 2010). In cortical neurons and vascular smooth muscle cells, the ER membrane protein STIM1 (**Figure 1.1**), which activates ORAI channels to promote SOCE, attenuates calcium entry through the L-type VGCC $Ca_V1.2$ (C. Y. Park et al., 2010; Y. Wang et al., 2010). In the reverse direction, changes in membrane potential can alter calcium conductance through ORAI channels (Bakowski & Parekh, 2000). This bidirectional regulation may enable preferential utilization of store-operated or activity-dependent calcium entry in different cell types. In collaboration with calcium buffers, pumps and transporters, such crosstalk tunes calcium dynamics to control intracellular signaling. Much remains to be understood, however, about mechanisms coordinating calcium dynamics in the developing cortex to preferentially activate specific downstream transcriptional pathways.

1.3.3 Alternative splicing regulates calcium entry and homeostasis

Differentiation in the embryonic cortex is accompanied by global splicing changes (X. Zhang et al., 2016). Moreover, in different cell and tissue types, alternative splicing of calcium channels and calcium-dependent effectors regulates calcium signaling by generating functionally diverse isoforms (Lipscombe et al., 2013). Precise isoform utilization in the embryonic cortex may, therefore, be an important contributor to cell- and region-specific calcium responses to developmental stimuli.

Alternative splicing of VGCC transcripts yields channel variants with specialized roles in different tissues, cells and cellular compartments (Abernethy & Soldatov, 2002; Lipscombe et al., 2013). *CACNA1C* (encoding the pore-forming subunit of the VGCC $Ca_v1.2$), for example, is extensively spliced to generate channels with distinct properties (Soldatov, 1994; Soldatov et al., 1997; Tang et al., 2004), and long-read sequencing confirms region-specific *CACNA1C* splicing in the adult human brain (Clark et al., 2020). Stereotyped utilization of specific exons also yields dominant tissue-specific *Cacna1c* isoforms, suggesting the presence of coordinated splicing events (Tang et al., 2007; Welling et al., 1997). Disease-causing mutations restricted to specific isoforms could thus give rise to channelopathies reflecting their expression pattern (Abernethy & Soldatov, 2002; Lipscombe et al., 2013).

Calcium channel splicing is also temporally regulated. Our studies and others point to developmental switches in *CACNA1C/Cacna1c* exon utilization in the brain, implying roles for different channel isoforms over time (Panagiotakos et al., 2019; Tang et al., 2009, 2011). For instance, utilization of two mutually exclusive *CACNA1C* exons (*8* and *8a*), which are mutated in the syndromic autism spectrum disorder (ASD) Timothy syndrome (TS), is developmentally controlled (Panagiotakos et al., 2019; Tang et al., 2011). In addition to impeding channel inactivation (Splawski et al., 2004), the TS mutation in *exon 8a* prevents a normal developmental splicing switch in patient cells (**Table 1;** (Panagiotakos et al., 2019; Tang et al., 2011)). This results in continued mutant channel expression in developing neurons and ensuing cellular phenotypes contributing to TS (Birey et al., 2017, 2022; Panagiotakos et al., 2019; Paşca et al., 2011); **Table 1**, discussed in more detail later). How channel subunit isoforms associate with one another, and how their expression is regulated across time, is unclear but likely influences cell type-specific calcium responses.

Tissue- and maturation state-specific PMCA isoform expression has also been reported (Brandt & Neve, 1992; Kip et al., 2006). Both neural activity and calcium regulate PMCA splicing (Carafoli et al., 1999; Zacharias & Strehler, 1996), supporting that activity-dependent feedback influences calcium signaling via splicing regulation. Splice variants of many other regulators of calcium homeostasis have been identified, including N-methyl-D-aspartate receptors (NMDARs) (An & Grabowski, 2007; Vallano et al., 1999), ORAI1 (Fukushima et al., 2012), STIM1 (Darbellay et al., 2011; Ramesh et al., 2021), and calcium-dependent transcription factors like NFAT (Vihma et al., 2008) and CREB (Walker et al., 1996). Mapping the cell type-specific expression of calcium channel and signaling protein isoforms across development is essential to understanding how calcium elicits specific responses in the embryonic cortex.

1.4 – Calcium signaling is deregulated in neurodevelopmental disorders

Mutations in genes impinging on calcium signaling have been implicated in neuropsychiatric disorders of developmental origin. In this section, we discuss genetic evidence highlighting that disrupted calcium-dependent molecular networks may contribute to misregulation of cellular behaviors in the developing brain. To aid our discussion, we surveyed the Simons Foundation Autism Research Initiative (SFARI) Gene database in 2022 for studies implicating regulators of calcium signaling in the etiology of neurodevelopmental disorders. We restrict our analysis (summarized in **Table 1** and discussed below) to genes encoding proteins that either: 1) directly regulate calcium entry, signaling or homeostasis, or 2) indirectly modulate calcium signaling by altering membrane potential or excitability.

Genetic studies have associated mutations in VGCC subunits with increased risk for neuropsychiatric disorders (**Table 1**). For example, *CACNA1C* variants are associated with bipolar

disorder, schizophrenia and ASD (Bhat et al., 2012; Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). Classical TS, a syndromic ASD, is caused by a point mutation in *CACNA1C* (**Table 1**). The TS mutation impairs both voltage-dependent $Ca_v1.2$ channel inactivation, resulting in elevated depolarization-induced calcium, and channel splicing, leading to persistent mutant channel expression in immature neurons (Panagiotakos et al., 2019; Paşca et al., 2011; Splawski et al., 2004). The ensuing abnormalities in channel signaling yield a constellation of cellular phenotypes, including differentiation deficits, activity-dependent dendritic retraction and impaired interneuron migration (Birey et al., 2017; Krey et al., 2013; Panagiotakos et al., 2019; Paşca et al., 2011). Mutations in genes encoding other calcium-permeable channels, such as the GluN2 NMDAR subunits, have also been linked to neurodevelopmental conditions (Endele et al., 2010) (**Table 1**). In addition, variants of *ATP2B2* (Iossifov et al., 2014), which encodes the PMCA2 calcium pump, and missense mutations in *ITPR1,* which encodes the IP3 receptor type 1 (IP3R1), are implicated in ASD (De Rubeis et al., 2014; Iossifov et al., 2014; T. Wang et al., 2016). In line with this, fibroblasts from individuals with syndromic and sporadic ASD display attenuated IP3-dependent calcium signaling (Schmunk et al., 2015, 2017).

Mutations in other ion channels and neurotransmitter receptors, which can indirectly influence voltage-dependent calcium influx, are also associated with neurodevelopmental disorders. Genetic variants in *SCN2A*, encoding the $Na_v1.2$ sodium channel, for example, are strongly linked to infantile epilepsy, ASD, and intellectual disability (ID) (Sanders et al., 2018) (**Table 1**). One such mutation (K1422E) in $Na_v1.2$ channels renders them calcium-permeable. $Scn2a^{K1442E/+}$ cortical neurons display larger action potential-evoked calcium transients compared to wild-type neurons, suggesting that this mutation impacts calcium signaling (Echevarria-Cooper et al., 2021). Similarly, potassium channel mutations that alter excitability (e.g., *KCNQ2* variants)

and chromosomal abnormalities in loci containing γ-aminobutyric acid receptor (GABAR) genes (e.g., *15q11-13*) have been associated with neurodevelopmental phenotypes (**Table 1**) (Cook et al., 1998). Imbalances in GABAergic and glutamatergic signaling are postulated to contribute to the etiology of neurodevelopmental disorders (Sohal & Rubenstein, 2019), and a recent study identifying genes with altered expression trajectories in ASD further hints at critical roles for ion channels and GABAergic neurons in ASD pathophysiology (Berto et al., 2022).

In addition to ion channel mutations, activity-dependent transcriptional regulation, which is critically dependent on calcium, has been implicated in neurodevelopmental psychiatric disorders (Boulting et al., 2021; Sanchez-Priego et al., 2022). Altered activity-dependent splicing networks have also been reported in ASD cohorts (Gandal et al., 2018; Parikshak et al., 2016; Quesnel-Vallières et al., 2016). Finally, even in instances where known calcium signaling effectors are not mutated, alterations in calcium handling have been observed in cells from individuals with neurodevelopmental conditions. For example, cortical organoids from patients with *22q11.2* deletion syndrome, a highly penetrant cause of neuropsychiatric disease, exhibit calcium signaling deficits (Khan et al., 2020). Transplanted human induced pluripotent stem cell (iPSC)-derived astrocytes from ASD individuals also display elevated calcium responses (Allen et al., 2022). These observations suggest that calcium signaling may be a convergence point for multiple developmental neuropsychiatric disorders, highlighting a need for understanding its contributions to the execution of cellular behaviors during development.

Table 1.1 – Developmental disease-associated mutations intersect with calcium signaling

Select genes from the SFARI Gene Database (see: https://gene.sfari.org/about-gene-scoring/ for the SFARI gene scoring criteria) encode protein products that either directly regulate calcium homeostasis and signaling or indirectly impinge on calcium by modulating neuronal excitability or altering membrane potential. We restricted our survey to genes defined as either Category 1 (high confidence) or Category 2 (strong candidate) from the SFARI gene database in 2022. We list here the developmental functions of their gene products and the functional effects (when known) of select disease-associated mutations. This Table only highlights selected genes and is not exhaustive in scope. Notably, due to space constraints, we have not included synaptic structural proteins that regulate ion/calcium channel localization and function at synapses (e.g., ANK2 (Kline et al., 2014), NRXN1-3 (Luo et al., 2020; Missler et al., 2003)), which have been reproducibly implicated in ASD.

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
CALCIUM CHANNELS					
ATP2B2	missense, nonsense, truncating variants,	ASD, epilepsy	encodes the PMCA2 ATPase calcium pump	> LoF mutations predicted to result in excess intracellular calcium; > ASD associated variants (e.g., Cys756*, Trp1064* - predicted to lead to nonsense mediated decay); > heterozygous LoF mice: heightened amplitude AMPAR-mediated calcium transients in cerebellar Purkinje cells; increased expression of P/Q-type VGCCs; increased amplitude depolarization-induced calcium influx, decreased Purkinje cell numbers; > KO mice: reduced growth rate, balance and movement problems, deafness and vestibular problems	***Select genetic studies:*** *Carayol et al. 2011; Takata et al., 2018; Iossifov et al. 2014; Yang et al., 2013; Prandini et al., 2012* ***Select functional references:*** *Smits et al., 2019; Fakira et al., 2012; Kozel et al., 1998*
CACNA1A	missense, frameshift, stop-gain and LoF variants	ASD, ID, epilepsy (e.g., EE, primary generalized epilepsy), paroxysmal movement disorders; FHM	encodes the pore forming subunit of the VGCC Cav2.1	> GoF mutations (e.g. R192Q mutation implicated in FHM): knock-in mice exhibit elevated calcium current density in cerebellar granule cells, increased neurotransmitter release at neuromuscular synapses, enhanced induction of cortical spreading depression; > epilepsy related mutations: dominant negative, impaired channel function (e.g. C5733T, E147K); > KO mice: ataxia, dystonia and early death, irregular corticothalamic activity; > interneuron-specific LOF: epilepsy, abnormal GABA release from PV+ cells; > pyramidal neuron specific LOF: attenuates excitability	***Select genetic studies:*** *Gulsuner et al., 2020; Epi4k Consortium 2016; Hamdan et al., 2017; Damaj et al., 2015; Jiang et al., 2015; Jouvenceau et al., 2001; Imbrici et al., 2004; Stam et al., 2009; Noebels et al., 2002* ***Select functional references:*** *Bomben et al., 2016; Rossignol et al., 2013; Llinás et al., 2007; Todorov et al., 2006; Maagdenberg et al., 2004; Kaja et al., 2005; Noebels et al., 2002*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
CACNA1C	missense variants, G406R point mutation in *exon 8a*	classical TS, SCZ, BPD	encodes the pore forming subunit of the VGCC Cav1.2	> GoF TS mutation: impaired channel splicing, loss of voltage-dependent channel inactivation, increased depolarization-dependent calcium elevations, altered cortical projection neuron differentiation, activity-dependent dendritic retraction, deficits in cortical excitatory and inhibitory neuron migration	***Select genetic studies:*** *Splawski, et al. 2004; SCZ Psychiatric GWAS consortium 2011; Cross-disorder group of Psychiatric Genomics Consortium, 2013; SCZ Working Group of the Psychiatric Genomics Consortium, 2014; Ripke, et al. 2013; Ferreira et al, 2008; Purcell, et al. 2014, Green et al., 2013* ***Select functional references:*** *Splawski, et al. 2004; Barrett & Tsien, 2008; Pasca et al., 2011; Krey et al., 2011; Birey et al., 2017; Kamijo et al., 2018; Panagiotakos et al., 2019; Bhat et al., 2012*
CACNA1D	missense, LOF, and GoF variants	ASD, epilepsy	encodes the pore forming subunit of the VGCC Cav1.3	> GoF mutations: impaired/irregular voltage-dependent channel inactivation (e.g., G407R, G403D, G403R, Q547H, P1336R, A769G, V401L and S652L); hyperpolarized voltage dependence of activation (e.g., V259D, F747L, I750M, V1153G, A769G, V401L and S652L); increased current density (e.g. V401L); > LoF mutations: increased sensitivity to dihydropyridine inhibition (e.g. S652L); > ASD-associated mutation A760G: reduced calcium dependent inactivation, altered channel gating leading to increased intracellular calcium > KO mice: deafness, cardiac irregularities	***Select genetic studies:*** *O'Roak et al., 2012; Klassen et al., 2011* ***Select functional references:*** *Pinggera et al., 2015, 2017, 2018; Hofer et al., 2020; Ortner et al., 2020; Baig et al. 2011; Scholl et al. 2013; Limpitikul et al., 2016; Platzer et al., 2000*
CACNA1E	missense, synonymous, and GoF/LoF variants	ASD, DEE	encodes the pore forming subunit of the VGCC Cav2.3	> G1209S: top de novo ASD risk mutation; > GoF mutations: slow inactivation, hyperpolarizing shifts in voltage dependent activation, increased current density (e.g. majority of DEE variants in pore domains, S6 variants: F698S, I701V, A702T; S4-5 variant: I603L); > KO mice: CA1 pyramidal cells from KO mice exhibit increased excitability, and altered action potential properties; neurons of the reticular thalamus have significantly reduced high voltage activated calcium currents, and suppressed rhythmic burst discharges.	***Select genetic studies:*** *O'Roak et al., 2012; Takata et al., 2016; Helbig et al., 2018; Carvill et al., 2019; Neale et al., 2012* ***Select functional references:*** *Helbig et al., 2018, Zaman et al., 2011, Neale et al., 2012; Carvill et al., 2019; Gutzman et al., 2019*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
CACNA1H	missense and LoF/GoF variants	ASD, epilepsy (e.g. CAE)	encodes the pore forming subunit of the VGCC Cav3.2	> missense LoF ASD mutations in voltage sensor (e.g., R212C, R902W), pore-forming domains (e.g., W962C), and c-terminus (e.g., R1871Q + A1874V): significantly reduced Cav3.2 (T-type) calcium currents; > CAE associated variants result in GoF and LoF (e.g., C456S, G773D, R788C, V831M, E282K: altered voltage dependence of activation; F161L, P648L, G773D, R788C: altered voltage dependence of inactivation; C456S, A748V, D1463N: faster activation kinetics; G773D, G784S, R788C, V831M: slower activation kinetics; F161L: faster inactivation kinetics; G499S, P648L, G773D, R788C, V831M, G773D, V831M, G848S, R788C, G773D, R788C: slower inactivation/deactivation kinetics); > modeling predicts net GOF/enhanced burst firing of thalamic neurons for CAE associated variants (e.g., C456S, P648L, G773D, R788C, G773D-R788C, A748V, G784S, G848S, and D1463N); > modeling predicts decreased thalamic neuron burst firing for CAE associated variants (e.g., E282K and V831M); > KO mice have anxiety-like phenotypes and impairments in memory	***Select genetic studies:*** *Chen et al., 2003; Splawski et al., 2006; Vitko et al., 2005; Heron et al., 2007* ***Select functional references:*** *Khosravani et al., 2004; Perez-Reyes 2006; Vitko et al., 2005; Vitko et al., 2007; Heron et al., 2007; Gackiere et al., 2008, Tao et al., 2008; Becker et al., 2008; Powell et al., 2009; Hu et al., 2009; Gangarossa et al., 2014; Splawski et al., 2006*
CACNA2D3	missense variants, splice site mutations, deletions	ASD, epilepsy	encodes the auxiliary α2δ3 VGCC subunit	> deletion of *CACNA2D3* homolog *unc-36* in c-elegans: altered axon termination; > *Cacna2d3* KO mice: impaired hearing, increased anxiety-related behaviors, reduced sensitivity to thermal pain	***Select genetic studies:*** *Iossifov et al., 2012; Girirajan et al., 2013; De Rubeis et al., 2014; Yuen et al., 2017* ***Select functional references:*** *Buddell et al., 2019; Peng et al., 2021; Landmann et al., 2019; Pirone et al., 2014; Neely et al., 2010*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
CACNB2	various missense variants	ASD	encodes the auxiliary β2 VGCC subunit	> GoF ASD-associated mutations (e.g., G167S, S197F): slower time dependent inactivation, altered sensitivity of voltage dependent inactivation, enhanced peak current densities; > LoF ASD-associated mutations (e.g., F240L): accelerated time-dependent inactivation	***Select genetic studies:*** *Cross disorder group of the psychiatric genomics consortium 2013; Ripke et al., 2013; Cross disorder group of the psychiatric genomics consortium 2017; Pardinas et al., 2018, Yuen et al., 2015* ***Select functional references:*** *Breitenkamp et al., 2014; Graziano et al, 2021; Despang et al., 2020*
TRPC6	translocations, missense variants, premature stop variant	ASD	encodes a calcium permeable cation channel from the transient receptor potential family	> neural progenitor cells from ASD patient-derived iPSCs: reduced calcium influx; > neurons from ASD patient-derived iPSCs: reduced TRPC6 protein levels, reduced dendritic spine density, altered synaptic properties; > transgenic *Trpc6* GOF: increased dendritic spine density, enhanced spatial memory	***Select genetic studies:*** *Griesi-Oliveira et al., 2014* ***Select functional references:*** *Griesi-Oliveira et al. 2015; Zhou et al., 2008*
TRPM1	CNVs, deletions	ASD	encodes a calcium permeable cation channel from the transient receptor potential family	> *Trpm1* KO mice: hyperactive, attenuated anxiety-related behaviors, altered fear memory, abnormal social behavior	***Select genetic studies:*** *Girirajan et al., 2013; Matsunami et al., 2014* ***Select functional references:*** *Hori et al., 2021*
OTHER ION CHANNELS THAT IMPINGE ON CALCIUM ENTRY AND ACTIVITY DEPENDENT SIGNALING					
CHRNA7	copy number variants, missense variants, microdeletion	Epilepsy, SCZ, DD, ID, ASD	encodes a calcium permeable nicotinic cholinergic receptor	> KO mice: phenotypes dependent on background strain due to differences in endogenous *Chrna7*; > C3H KO mice: impaired LTP, abnormal auditory processing; > C3H Het mice: reduced GAD65 levels, reduced GABAA receptors in male mice; > reduced *CHRNA7* transcription and function (e.g., *CHRNA7* promoter variants); decreased calcium influx through CHRNA7 (e.g., both 15q13.3 deletions and duplications); increased ER stress (e.g., 15q13.3 duplications)	***Select genetic studies:*** *Mikhail et al., 2011; Shinawi et al,. 2009; Soler-Alfonso et al., 2014; Gillentine et al., 2017; Hoppman-Chaney et al., 2013; Leonard et al., 2002; Gault et al., 2003; Stephens et al., 2009* ***Select functional references:*** *Yin et al., 2017; Freund et al., 2016; Adams et al., 2012; Leonard et al., 2002; Sinkus et al., 2010; Gillentine et al., 2017*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
KCNJ10	missense variants, synonymous variants, LoF/GoF variants	Seizures, ASD	encodes an ATP-sensitive potassium channel	> KO mice: motor impairments, hypomyelination, axonal abnormalities, retarded growth, dehydration, abnormalities in renal salt handling, premature death; > astrocyte- and oligodendrocyte-specific cKO: seizures, premature death, ataxia, hindleg paralysis, retarded growth, abnormal astrocyte depolarization, impaired uptake of glutamate and potassium, reduced spontaneous neuronal activity, abnormalities in post-tetanic potentiation and short-term potentiation; > oligodendrocyte-specific cKOs: mitochondrial abnormalities, axonal degeneration, loss of axonal integrity, neuronal loss, visual deficits, retinal abnormalities, motor problems, functional abrnomalities including slow potassium clearance, delayed axonal recovery post □hosphor□it, seizures; > GoF mutations: enhanced membrane expression; increased current density (e.g., R18Q); pH-dependent current inhibition (e.g., R348H); increased current amplitudes (e.g., R18Q, V84M); increased unit conductance (e.g. V84M); > homozygous missense mutations affecting transmembrane domain (e.g., G65P, G77R) modeled in *Xenopus*: decreased potassium currents	***Select genetic studies:*** *Ferraro et al.,2004; Buono et al., 2004; Sicca et al., 2011; Bockenhauer et al., 2009* ***Select functional references:*** *Bockenhauer et al., 2009; Neusch et al., 2000; Djukic et al., 2007; Schirmer et al., 2018; Larson et al., 2018; Moro et al., 2011*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
KCNMA1	missense variant, frame shift variant, GoF variants, LoF variant	Epilepsy, ASD, paroxysmal dyskinesia	encodes the pore-forming α-subunit of the large conductance calcium and voltage-activated potassium channel	> LoF mutations: altered resting membrane potential, decreased channel activity; > KO mice: alterations in suprachiasmatic nucleus function and circadian rhythm, progressive hearing loss, cerebellar abnormalities, motor coordination dysfunction, reduced activity of Purkinje neurons, dysfunctional astrocyte/arteriolar smooth muscle communication; > GoF mutations: enhanced voltage dependent activation and enhanced voltage sensitivity (e.g. N536H); in *Xenopus* oocytes: increased voltage- and calcium-dependent activation, faster activation kinetics in response to depolarizing voltage (e.g., D434G mutation); in CHO cells: increased sensitivity to calcium, activation at lower voltages, increase in open-channel probility (e.g., D434G)	***Select genetic studies:*** *Laumonnier et al., 2006; Neale et al., 2012; Liang et al., 2019; Zhang et al., 2020; Satterstrom et al., 2020; Zou et al., 2021; Du et al., 2011; Bailey et al., 2019; Jiaping et al, 2017; Du et al., 2005; Tabarki et al., 2016; Zhang et al., 2015* ***Select functional references:*** *Plger et al., 2000; Dez-Sampedro et al., 2006; Meredith et al., 2006; Ruttiger et al., 2004; Sausbier et al., 2004; Filosa et al., 2006; Zhang et al., 2020; Du et al., 2005*
GRIA2	nonsense variant, missense variant, deletions	ASD, ID, seizures, DEE, DD	encodes the GluA2 AMPAR subunit, whose inclusion in AMPARs renders them calcium-impermeable	> LoF mutations: decrease current amplitudes compared to wild type (WT) channels (e.g., G47E, D302G, del528-530, G609R, A639S, F644L, T646N); > GoF mutations: increase current amplitudes compared to WT channels (e.g., Q607E); > KO mice: increased calcium permeability in hippocampal neurons lacking GluA2, alterations in LTP, impaired exploratory behavior, alterations in reward behaviors	***Select genetic studies:*** *De Rubeis et al., 2014; Deciphering Developmental Disorders Study; Yuen et al., 2017; Hackmann et al., 2012* ***Select functional references:*** *Salpietro et al., 2019; Lu et al., 2009; Jia et al., 1996; Hackmann et al., 2012; Mead et al., 2003*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
GRIN1	missense variants	ASD, Polymicrogyria, Epilepsy, ID, DD	encodes the GluN1 subunit of calcium permeable NMDARs	> LoF missense variants in or adjacent to a transmembrane domain (e.g., P557R, S560dup, G618R, G620R, Y647S, G815R, F817L, Q556*, G827R); > GoF mutations: increased calcium currents through NMDARs (e.g., E662K); > *Grin1* KO mice: significantly attenuated NMDA-induced calcium rises, abnormal social behaviors; > *in utero* knockdown in developing cortex: deficits in radial migration; > conditional *Grin1* deletion in PV+ cells: reduced LTP induction and maintenance, impaired social behavior; > interneuron-specific *Grin1* deletion: attenuated NMDA currents; > conditional *Grin1* deletion in pyramidal cells: increased social approach behavior; > *Dlx5/6*-Cre mediated deletion *in utero*: abnormal morphological □hosphor□i of Re+ interneurons and improper circuit integration	***Select genetic studies:*** *Epi4K Consortium et al., 2013; Hamdan et al, 2011* ***Select functional references:*** *Hamdan et al, 2011; Gandal et al., 2012; Saunders et al., 2013; Lemke et al. 2016; Lemke 2020; Jiang et al., 2015; Forrest et al., 1994; Ferri et al., 2020; Billingslea et al., 2014; De Marco Garcia et al., 2015; Fry et al., 2018; Vieira et al,. 2021*
GRIN2A	copy number variants, nonsense mutation, chromosome translocation breakpoints, missense variant	ASD, various epileptic syndromes (e.g., Landau-Kleffner syndrome, EE, childhood epilepsy, infantile onset EE), polymicrogyria, SCZ	encodes the GluN2A subunit of calcium permeable NMDARs	> GoF mutations: increased GluN1/GluN2A current density (e.g., R1067W); increased sensitivity to glutamate and/or glycine agonists (e.g., P552R, M817V, L812M, V452M, K669N, N447K, V506A, P699S); decreased magnesium block, increased current density (e.g., N447K); impaired zinc inhibition, normal current amplitude, glutamate and glycine affinities and open-state probabilities (e.g., A243V); increased total surface expression (e.g., K590N, K879R, R1067W); > LoF mutations (e.g., R518H, T531M, D731N, V685G, G483R, A716T, C436R, C231Y, A548T, P79R, I694T, M705V, A727T, V734L, K772E, R370W); loss of magnesium block and decreased calcium permeability (e.g., N615K); > *Grin2A* KO mice: altered NMDA/AMPA receptor currents in hippocampal neurons, impairments in LTP and LTD, increased locomotion, deficits in fear conditioning; > *in utero* knock down: no effect of cortical radial migration	***Select genetic studies:*** *Barnby et al., 2005; Endele et al 2010; Lemke et al., 2013; Yoo et al., 2012; Liu et al., 2021; Singh et al., 2020 (medRxiv)* ***Select functional references:*** *Liu et al., 2021; Endele et al 2010; Strehlow et al., 2018; Kannagara et al., 2015; Sakimura et al. 1995; Kiyama et al. 1998; Chen et al., 2017; Ogden et al., 2017; Yuan et al., 2014; Marwick et al., 2019; Bertocchi et al., 2021; Swanger et al., 2016; Xu et al., 2018; Swanger et al., 2016; Gao et al., 2017; Addis et al., 2017; Ogden et al., 2017; Serraz et al., 2016*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
GRIN2B	splice site variants, non-synonymous variants, LoF variants, *GRIN2B* GoF (West syndrome), chromosome translocation breakpoints, frameshift mutations, missense mutations,	ASD, SCZ, West Syndrome, ID, DD, Epilepsy	encodes the GluN2B subunit of calcium permeable NMDARs	> GoF mutations: loss of voltage dependent magnesium block (e.g., N615I, V618G); reduced calcium permeability (e.g., N615I); > LoF GluN2B-724 truncation mutant: attenuated calcium influx, impaired trafficking to cell membrane, impaired dendritic morphogenesis; > LoF mutations: mutation in ligand binding domain, impaired glutamate binding, higher glutamate concentrations required for activation (e.g., G689S, G689C), poor expression of G689C variant at cell membrane; overexpression in hippocampal neurons: decreased frequency of NMDAR-dependent mEPSCs with faster deactivation kinetics, no change in frequency of AMPAR-dependent mEPSCs; > KO GluN2B to GluN2A "replacement" mouse: altered synaptic plasticity/development, reduced social exploration, hyperlocomotion; > MADM-mediated *Grin2*b KO: impaired dendritic pruning and patterning in DG granule cells and spiny stellate cells; > Dlx5/6-Cre mediated *Grin2b* deletion: abnormal morphological maturation of Re+ interneurons > GluN2B overexpression in mice: altered synaptic function, enhanced memory/learning	***Select genetic studies:*** *De Rubeis et al., 2014; Iossifov et al., 2015; Ohtsuki et al., 2001; Myers et al., 2011; Lemke et al., 2014; Epi4K Consortium et al., 2013; Endele et al., 2010; Zhao et al.,2011; Tarabeux et al., 2011; O'Roak et al., 2011; O'Roak et al., 2012; Kellner et al., 2021; Jiang et al., 2015* ***Select functional references:*** *Lemke et al., 2014; Swanger et al., 2016; Tang et al., 1999; Freunscht et al., 2013; Fedele et al., 2018; Akashi et al,. 2009; Wang et al., 2009; Wang et al., 2011; Kellner et al., 2021; Bahry et al., 2021; Sceniak et al., 2019; De Marco Garcia et al., 2015; Espinosa et al., 2009*
GABRB2	missense variants, LoF mutations	ASD, epilepsy (e.g., CAE, DEE, EME)	encodes the β2 subunit of the GABAA receptor, activation elicits calcium influx in cortical NSPCs	> LoF disease-associated variants in N-terminal and transmembrane regions: reduced GABA-induced current amplitudes (e.g., I246T, V282A, I288S, P252L); > G*abrb2* KO mice: increased susceptibility to seizures and epilepsy, altered startle response, reduced anxiety/depression behaviors, hyperlocomotivity, social behavioral abnormalities, memory deficits	***Select genetic studies:*** *Srivastava et al., 2014; Iossifov et al. 2014; Satterstrom et al., 2020; Yang et al., 2020; Hamdan et al., 2017* ***Select functional references:*** *Achkar et al, 2021; Yeung et al., 2018*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
GABRB3	de novo LoF mutations, missense mutations, intronic variants, synonymous mutations	ASD, epilepsy (e.g., CAE)	encodes the β3 subunit of the GABAA receptor; activation elicits calcium influx in cortical NSPCs	> LoF CAE variants: reduced current densities compared to wild type channels (e.g., P11S, S15F, G32R); reduced peak current amplitudes and reduced surface expression (e.g., P11S)	***Select genetic studies:*** *Epi4K Consortium et al., 2013; Chen et al. 2014; De Rubeis et al., 2014; Yang et al., 2017; Urak et al., 2006; Tanaka et al., 2008; Sanders et al., 2015* ***Select functional references:*** *Delahanty et al., 2012; Urak et al., 2006; Tanaka et al., 2008; Jiang et al., 2015; Tanaka et al., 2012*
KCNB1	LoF mutations, missense mutation, nonsense variant	DD, epilepsy (e.g., EE, West Syndrome), ASD	encodes the voltage-gated potassium channel Kv2.1	> V378A: altered subcellular localization, altered electrophysiological properties (retained voltage dependent activation with faster inactivation kinetics), altered ion selectivity; > EE-associated mutations (e.g. S347R, G379R, T374I): deficits in ion selectivity; > epilepsy-associated mutations (e.g. R306C, G401R): R306C – altered voltage sensitivity, reduced neuronal firing; G401R – dominant negative mutation in channel pore, G401R abolishes Kv2.1 currents and reduces neuronal firing; > *Kcnb1* KO mice: hyperactivity, abnormalities in spatial learning, accelerated progression of seizures	***Select genetic studies:*** *de Kovel et al., 2017; Torkamani et al., 2014; Parrini et al., 2017; Thiffault et al., 2015; Srivastava et al., 2014; Allen et al., 2016; Saitsu et al., 2015* ***Select functional references:*** *Torkamani et al., 2014; Thiffault et al., 2015; Speca et al., 2014; Saitsu et al., 2015*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
KCNQ2	splice site variant, LoF mutations, missense variants, frameshift variants	ASD, BPD, epilepsy (e.g. DEE, benign familial neonatal seizures)	encodes the voltage gated potassium channel Kv7.2	> LoF mutations largely implicated in BFNS; > voltage sensor S4 transmembrane segment mutations: impaired channel function, dominant negative effect at subthreshold voltages, depolarizing shift in activation curve (e.g., I205V and R213Q; R213Q and I205V); reduced current amplitudes, increased channel protein levels (e.g., R213Q); > pore forming S5 and S6 domain mutations: impaired channel function, dominant negative (e.g., A265P, T274M, G290D); > c-terminal mutations: reduced potassium current amplitude (e.g., R532W, M518V); > *Kcnq2* heterozygous null mice (modeling LoF pathogenic mutations): increased susceptibility to seizures and behavioral abnormalities including reduced sociability and hyperactivity; > homozygous null *Kcnq2* LOF: increased pyramidal neuron excitability	***Select genetic studies:*** *Singh et al., 1998; Jiang et al., 2013; Milh et al., 2013; Judy et al., 2013, Borsotto et al., 2007; Lee et al., 2019* ***Select functional references:*** *Biervert et al., 1998; Borsotto et al., 2007, Cook 1998; Zhang et al., 2020; Soh et al., 2014; Jiang et al., 2015*
KCNQ3	translocation mutations, missense variant	ASD, epilepsy (e.g., benign familial neonatal seizures)	encodes the voltage gated potassium channel Kv7.3	> LoF variants largely implicated in BFNS; > GoF mutations linked to ASD and DD: channel GOF, not associated with seizures in patients (e.g., R230C, R230H, R230S, R227Q); voltage sensor mutations (e.g., R227 and R230); > LoF mutations: voltage sensor mutations (e.g., M240R; homomeric channels not functional, mutation affects voltage sensitivity, predicted increase in channel resting state stability and destabilization of active state); significantly reduced current density, impaired channel function (e.g., R330L, R330C)	***Select genetic studies:*** *Hirose et al., 2000; Epi4K Consortium et al., 2013; Miceli et al., 2020; Grinton et al.,2015; Gilling et al., 2013* ***Select functional references:*** *Charlier et al., 1998; Micelli et al., 2015; Miceli et al., 2020; Gilling et al., 2013; Sands et al., 2019;*
P2RX5	gene disrupting mutations, LoF variants	ASD	encodes a calcium permeable purinergic receptor	unknown?	***Select genetic studies:*** *Sanders et al., 2015; Iossifov et al. 2014, Hu et al., 2006* ***Select functional references:*** *Bo et al., 2003*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
SCN1A	protein truncation mutations (LoF), frameshift, nonsense, splice-donor, missense mutations	Asperger Syndrome, ASD, FHM, epilepsy (Dravet Syndrome, Severe myoclonic epilepsy of infancy, GEFS+)	encodes the α subunit of the voltage-gated sodium channel Nav1.1	> *SCN1A* LoF mutations: Dravet syndrome; > LoF mutations: reduced current amplitude, hypersensitivity to steady-state inactivation (e.g., S1328P); abolished sodium currents in HEK cells (e.g., E78X, W384X, E1587K, and R1596C); partial LOF, abnormal recovery after inactivation, reduced sodium current density (e.g., E788K and M909K); slow inactivation recovery, hyperpolarizing shifts in activation/inactivation (e.g., D249E); reduced current density, slow recovery after inactivation (e.g., T1934I), slow recovery from inactivation (e.g., E78D); > interneurons from iPSCs derived from individuals with Dravet Syndrome (heterozygous S1328P mutations): sodium current and AP firing deficits; no electrophysiological abnormalities in excitatory neurons; > mutations causing GEFS+ mainly missense mutations; > select mutations linked to GEFS+ (functional effects influenced by cell type/expression system): increased Nav1.1 channel function in HEKs (D188V), increased Nav1.1 function in *Xenopus* oocytes (W1204R, R1648H, D1866Y); decreased Nav1.1 channel function in *Xenopus* oocytes (R859C, T875M); decreased Nav1.1 channel function in tsA201 (V1353L, I1656M, R1657C, A1685V); > LoF in mice: epileptic seizures	***Select genetic studies:*** *Weiss et al. 2003; Escayg et al., 2000; Epi4K Consortium et al., 2013; Claes et al., 2001; Osaka et al., 2007, Weiss et al., 2003, O'Roak et al,. 2011, O'Roak et al., 2012, De Rubeis et al., 2014, Yuen et al., 2017; Hamdan et al., 2017; Jiang et al., 2015* ***Select functional references:*** *Weiss et al., 2003; Ogiwara et al., 2007; Escayg and Goldin et al., 2010*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
SCN2A	LoF variants (often associated with ASD), missense variants, GoF variants, protein truncating variants (ASD)	ASD, ID, epilepsy (infantile EE, benign infantile seizures), SCZ	encodes the α subunit of the voltage gated sodium channel Nav1.2	> Effects of mutations differ in mature and immature neurons owing to changes in subcellular distribution and splicing; > LoF mutations: often associated with ASD and ID, impaired excitability in immature neurons and altered dendritic dendritic excitability in mature neurons; > GoF mutations: mostly associated with infantile epilepsies, increased neuronal excitability; > K1422E: mutation in selectivity filter, renders channel calcium permeable; > R1902C: ASD-associated mutation, allows calcium-dependent conformational change in Nav1.2 when complexed with CaM; > homozygous KO mice: perinatal lethality; > heterozygous *Scn2a* LoF mice: impaired excitability and action potential initiation in immature neurons; impaired dendritic excitability and disrupted synaptic function and plasticity in mature neurons, with presence of immature dendritic spines; normal PV and SST neuron Aps; hyperactivity, absence-like seizures, and learning delays	***Select genetic studies:*** *Hamdan et al., 2017; Jiang et al., 2015; Sanders et al., 2012; Tavassoli et al., 2014; Jiang et al., 2013; Sundaram et al., 2013; Carroll et al., 2016* ***Select functional references:*** *Begemann et al., 2019; Spratt et al., 2019; Ben-Shalom et al., 2017; Heinemann et al., 1992; Echevarria-Cooper et al., 2021; Fromer et al., 2014*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
SCN8A	various missense mutations	ASD, DD, ID, epilepsy (e.g. E1EE13)	encodes the voltage-gated α8-subunit of the sodium channel Nav1.6	> majority of patient mutations are GoF: increased channel activity due to alterations in opening and inactivation kinetics; > LoF mutations: cognitive impairment, movement disorders; > GoF mutations: heterozygous missense, increased neuronal excitability in postnatal rat hippocampal neuronal cultures (e.g., N1768D); enhanced channel activation in neurons in vitro, increased □hosphor□ity and spontaneous activity (e.g., T767I); altered voltage dependence of activation, predicted to yield premature neuronal firing (e.g., N984K); > heterozygous knock-in N1768D mice: seizures and Sudden Unexpected Death in Epilepsy, dose dependent effects (homozygous knock-in mice: earlier onset of seizures and death); > LoF mutations: abolishes sodium currents (e.g., G964R, E1218K); reduces protein abundance (e.g., E1218K); significantly reduced channel activity in HEKs, protein product stable and expressed at similar levels to WT, possible off target effects of mutant protein product (e.g., G1451S); > *in vitro* LoF but possible *in vivo* GoF (e.g, R223G: reduced current density and at 37C reduces protein stability; similar activation/inactivation kinetics as WT at 30C with increased ramp current – possible GoF > Conditional *Scn8a* deletion in inhibitory cells using Dlx5/6-Cre: absence epilepsy; no absence seizures with excitatory neuron-specific deletion	***Select genetic studies:*** *Epi4K Consortium et al., 2013; Veeramah et al., 2012, Blanchard et al., 2015; Estacion et al., 2014; de Kovel et al., 2014* ***Select functional references:*** *Veeramah et al., 2012; Wagnon et al., 2015; Blanchard et al., 2015; Estacion et al., 2014; Wagnon and Meisler 2015; de Kovel et al., 2014; Markinson et al., 2017*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
SLC6A1	truncating variants, missense variants, splice site variants CNVs, insertions, deletions and synonymous variants, nonsense variant	epilepsy (myoclonic atonic, genetic generalized, non-acquired focal, Lennox-Gastaut syndrome), DD, ASD, ID, speech delay, SCZ	encodes the voltage-dependent GABA transporter protein type 1 responsible for sodium-dependent GABA reuptake	> LoF mutations associated with Lennox-Gastaut syndrome: reduced total protein levels in HEKs and rat cortical neurons, reduced cell surface expression, impaired GABA uptake (e.g., G234S); > P361T: heterozygous LoF mutation associated with epilepsy and autism, lower total protein levels, mutant protein abnormally localized in ER, reduced protein function and surface expression; > KO mice: homozygous LoF yields increased tonic inhibition, reduced phasic inhibition, reduced quantal GABA release, no change in GABA receptor density, tremor, walking abnormalities, decreased strength, seizures, and anxious behaviors; heterozygous LoF yields diminished GABA reuptake	***Select genetic studies:*** *Sanders et al., 2012, Satterstrom et al., 2020, Satterstrom et al., 2019; Cai et al., 2019; Carvill et al., 2015; Rees et al., 2020; Carvill et al., 2015; Wang et al., 2020* ***Select functional references:*** *Cai et al., 2019; Carvill et al., 2015; Jensen et al., 2003; Cope et al., 2009; Chiu et al., 2005; Cope et al., 2009*
SELECT CALCIUM-INTERACTING PROTEINS, CALCIUM SIGNALING MODULATORS, AND ACTIVITY-DEPENDENT PROTEINS					
CREBBP	coding variants, point mutations, deletion, duplications, frameshift mutation, missense mutations, splice site mutations	ASD, Rubinstein-Taybi syndrome	encodes the calcium regulated transcriptional coactivator CBP, a CREB co-factor critical for activity-dependent gene expression	> CH1 domain mutant mice: deficits in social interaction, ASD-relevant repetitive behaviors, hyperactivity, and abnormal synaptic plasticity; > *Gfap*-cre mediated cKO mice: microcephaly, behavioral anomalies, reduced embryonic NSPC proliferation, deficits in NSPC migration, impaired postnatal neurogenesis, and abnormal pyramidal cell morphology	***Select genetic studies:*** *Petrij et al., 1995; Roelfsema et al., 2005; Barnby et al., 2005; Wincent, et al. 2016; Coupry et al., 2002* ***Select functional references:*** *Merk et al., 2018; Zheng et al., 2016; Schoof et al., 2019; Valor et al., 2011*
CASK	missense variants, splice site variant, premature stop variants	ASD	encodes a calcium/calmodulin-dependent serine protein kinase	> iPSC-derived neurons from *CASK* mutation carriers: reduced CASK levels, alterations in presynaptic development, reduced spontaneous calcium events; > neurons from *Cask* KO mice: increased frequency of spontaneous excitatory miniature synaptic events, decreased frequency of spontaneous inhibitory miniature synaptic events	***Select genetic studies:*** *Iossifov et al., 2014; Najm et al. 2008; Stessman et al. 2017; Becker et al., 2020; Mukherjee et al., 2020* ***Select functional reference:*** *Atasoy et al., 2007; Becker et al., 2020*
CIB2	copy number variants	ASD	encodes a calcium binding protein that mediates calcium signaling processes	> *Cib2* KO mice: hearing loss, loss of auditory hair cell currents; > CIB2 down regulation in zebrafish and *Drosophila*: deafness and vision deficits	***Select genetic studies:*** *Prasad et al., 2012; Riazuddin et al., 2012* ***Select functional references:*** *Wang et al., 2017; Riazuddin et al., 2012*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
DYRK1A	truncating variants, nonsense mutations, missense variants, frameshift mutations, splice site variants, copy number variants (DS), translocation	ASD, ID, microcephaly, DD	encodes a kinase that regulates localization and/or activity of calcium-dependent transcription factors (e.g. NFAT, CREB)	> *DYRK1A* in Down syndrome (DS) critical region, copy number increase implicated in DS; > LoF *DYRK1A* mutations: microcephaly, ID and ASD; > kinase domain mutations alter catalytic activity; R467Q: predicted function in protein stability; L245R: prevents DYRK1A autophosphorylation; > LoF mutations (e.g., E396ter: protein product degraded by ubiquitin proteasome pathway, inactive kinase, no dominant negative effect; other predicted LoF mutations: I48K(fs*2), A498P(fs*94), E414V(fs*76) (loss of exon 11, kinase domain mutation); > *in ovo* GoF in chick spinal cord: reduced proliferation; pharmacological LoF: increased proliferation and apoptosis; > embryonic overexpression upregulates p27Kip1 in the embryonic chick spinal cord and mouse cortex; > *in utero* GoF in mouse cortex: reduced proliferation, abnormal radial migration, enhanced neuronal differentiation, phenotypes dependent on timing and degree of over-expression; > Trisomy of *Dyrk1a* (in mBAC TgDyrk1a mice): lengthened G1 and RG cell cycle length, altered numbers of neurons and IPCs during embryonic neurogenesis; > *minibrain* (*Drosophila* ortholog): GoF promotes cell cycle exit, facilitates neuronal differentiation; haploinsufficiency results in microcephaly > *Dyrk1a* homozygous KO mice: growth delays, die at midgestation; *Dyrk1a* heterozygous ± mice: reduced brain size, increased neuronal density; > cortex-specific *Dyrk1a* deletion: decreased cortical mass, reduced neuronal size, disrupted growth factor signaling; heterozygous mutants: changes in axonal projections and deeper layer neuron morphology; increased ASD-related behaviors; > *in vitro* knockdown: altered dendritic growth and spine development	***Select genetic studies:*** *De Rubeis et al., 2014; Iossifov et al. 2015; Bronicki et al., 2015; Ji et al., 2015; Dang et al., 2018; Moller, et al. 2008; van Bon et al,. 2016; Courcet, et al. 2012; van Bon et al., 2011; Lee et al., 2020; Iossifov et al. 2015; Deciphering Developmental Disorders 2015; Iossifov et al., 2012; Ruaud et al., 2015* ***Select functional references:*** *Tejedor, 1995; Dang et al., 2018; Fotaki, et al. 2002; Lee et al., 2020; Yabut et al., 2010; Evers et al., 2017; Shaikh et al., 2016; Fotaki et al., 2004; Hammerle et al., 2011; Levy et al., 2021; Park et al., 2013; Guimera et al., 1996; Najas et al., 2015; Chakrabarti et al., 2007*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
MEF2C	nonsense mutation, deletions	ASD, DD, mental retardation	encodes a calcium-dependent member of the MADS box family of TFs	> genome wide analyses for TF binding sites: MEF2 controls many activity-dependent genes > LoF mutations in hiPSC-derived cortical neurons: aberrant electrophysiological properties, including elevated synaptic activity and increased excitation; > conditional *Mef2c* deletion in developing interneurons: reduced PV+ cell density, deficits in PV cell maturation; > *Mef2c* cKO in excitatory lineage: attenuated cortical network activity and ASD-, ID- and schizophrenia-related behaviors; > *Gfap*-Cre mediated *Mef2c* KO: increased excitatory synapse numbers, upregulated synaptic transmission, impairments in learning and memory; > *Mef2c* cKO in NSPCs (*Nestin*-cre): abnormal cytoarchitecture/compaction of cells in CP, altered cortical organization; > Mef2c KO mice: immature electrophysiological properties, reduced excitability; > expression of superactivating MEF2C: decreased frequency of mEPSCs	***Select genetic studies:*** *Novara et al., 2010; Neale et al., 2012* ***Select functional references:*** *Mayer et al., 2018, Allaway et al., 2021; Harrington et al., 2016; Trudler et al., bioRxiv 2020; Li et al., 2008; Barbosa et al., 2008; Flavell et al., 2008*

Gene	Mutation type	Disease	Relationship to calcium/activity dependent signaling	Electrophysiological characterization of select variants and/or select developmental phenotypes	References
RBFOX1	copy number variants	ASD, ID, epilepsy	encodes an RNA binding protein and activity-dependent splicing regulator	> encodes member of a family of splicing factors that regulate ion channels and synaptic proteins (e.g. *Cacna1c*); > 160kb deletion in first exon and intron: reduced expression of *Rbfox1* mRNAs; > knockdown: abnormal alternative splicing, abnormal neuroblast migration, impaired axon extension, impaired dendritic arborization, abnormal membrane and synaptic properties, increased neuronal excitability; > interneuron specific cKO: abnormal alternative splicing, increased PV- and SST-expressing interneuron density, impaired interneuron activity (i.e., decreased mIPSC frequency and amplitude, increased cFos expression), increased seizure susceptibility, altered synaptic connectivity; > CNS-specific knockdown: increased seizure susceptibility, increased neuronal excitability, and abnormal splicing	***Select genetic studies:*** *Hamada et al., 2016; Sebat et al., 2007; Martin et al., 2007; Bhalla et al., 2004; Mikhail et al., 2011; Davis et al., 2012; Zhao et al., 2013; Gandal et al., 2018* ***Select functional references:*** *Wamsley et al., 2018; Gehman et al., 2011; Martin et al., 2007; Bill et al., 2013; Tang et al., 2009*
SRCAP	de novo LoF variants, truncating variants, frameshift variants, missense variants	ASD, FHS	encodes an activator of the calcium-sensitive transcriptional coactivator CREBBP	> FHS variants map to last two exons of SRCAP (33 and 34), DNA methylation profiles vary depending on the location of the mutation	***Select genetic studies:*** *Hood et al, 2012; Iossifov et al., 2014; Stessman et al., 2017; Yuen et al., 2017; Rots et al., 2021; Alonso-Gonzalez et al., 2018*

** Footnote: Epileptic Encephalopathy (EE); Schizophrenia (SCZ); Intellectual Disability (ID); Developmental and Epileptic Encephalopathy (DEE); Childhood Absence Epilepsy (CAE); Developmental and Epileptic Encephalopathy (DEE); developmental delay (DD); Floating-Harbor syndrome (FHS); Timothy Syndrome (TS); Bipolar Disorder (BD); Episodic ataxia type 2 (EA2); Familial hemiplegic migraine (FHM); Generalized Epilepsy with Febrile Seizures Plus (GEFS+); early-infantile EE type 13 (EIEE13); early myoclonic encephalopathy (EME)*

Chapter 2 – Calcium-dependent regulation of cellular behaviors in the developing cortex

Chapter 2 is adapted from the following published article:

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https://journals.biologists.com/dev/article-lookup/doi/10.1242/dev.198853

Development of the cerebral cortex involves a series of spatiotemporally regulated cellular events, including neural stem and progenitor cell (NSPC) proliferation (Libé-Philippot & Vanderhaeghen, 2021; Y. Lin et al., 2021; Llorca & Marín, 2021), migration of newborn neuroblasts into the cortical plate (CP) (Francis & Cappello, 2021; Silva et al., 2019), and the differentiation of these cells into mature, synaptically active neurons and glia (Bonnefont & Vanderhaeghen, 2021; Taverna et al., 2014) (**Figure 2.1**). Spontaneous and agonist-induced calcium elevations, neurotransmitter- and depolarization-evoked calcium influx, and SOCE have been observed at various stages of embryonic and adult NSPC lineage progression (Toth et al., 2016; Uhlén et al., 2015). These different forms of calcium entry result in the induction of calcium-dependent transcriptional cascades (e.g., CREB-, MEF2-, NFAT, NPAS4-dependent gene expression) that contribute to developmental regulation of cellular behaviors (Greer & Greenberg, 2008). Achieving a granular picture of how cell type-specific calcium signaling impinges on

lineage progression in the developing cortex is critical for understanding normal development and neurodevelopmental disease. Below, we link calcium responses in developing cortical cell populations with the cellular machinery mediating these signals and controlling different cellular behaviors.

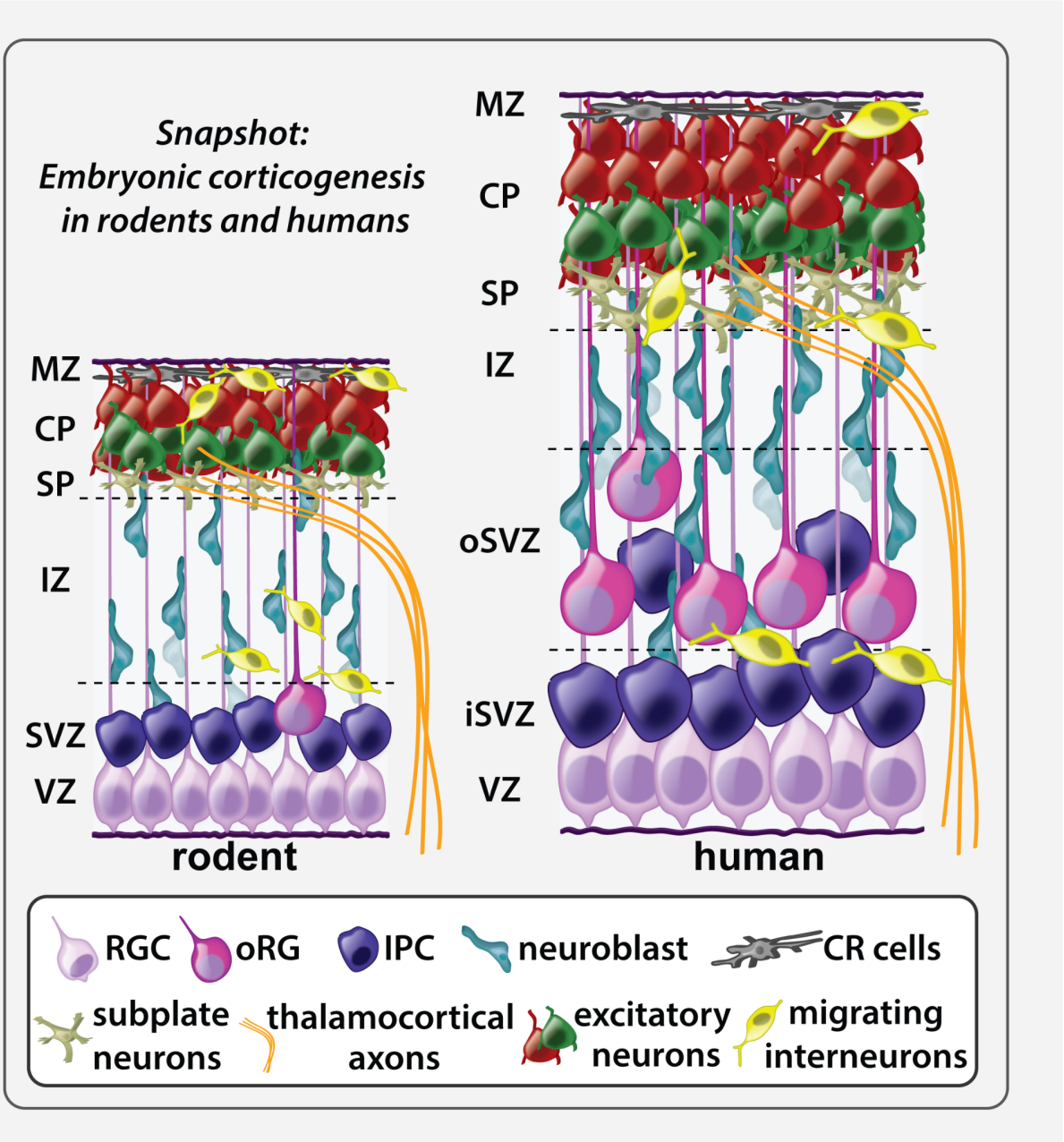

Figure 2.1 – Embryonic corticogenesis in rodents and humans
During early development, neuroepithelial cells give rise to radial glial cells (RGCs), proliferative neural stem cells that populate the cortex with neurons and astrocytes (Libé-Philippot & Vanderhaeghen, 2021; Y. Lin et al., 2021). RGCs initially divide symmetrically and subsequently switch to asymmetric divisions to generate postmitotic migratory neuroblasts and intermediate progenitor cells (IPCs; (Haubensak et al., 2004; Noctor et al., 2004)). Residing in the pseudostratified ventricular zone (VZ) adjacent to the ventricles, RGCs maintain contact with the overlying pia through a long radial fiber. Newborn neuroblasts exit the VZ and migrate along RGC fibers to reach their final laminar position. Newly generated IPCs detach from the ventricular surface and migrate into the subventricular zone (SVZ), where they divide symmetrically to produce daughter neurons. Young neurons sequentially exit the VZ/SVZ to build the cortex in an inside-out fashion, terminally differentiating in the cortical plate (CP) (Bonnefont & Vanderhaeghen, 2021). Early-generated subplate (SP) and Cajal-Retzius (CR) neurons, residing beneath the CP and in the marginal zone (MZ), respectively, are central to the development of cortical circuits (Hoerder-Suabedissen & Molnár, 2015; Kanold, 2019; López-Bendito, 2018). In particular, the transient SP population plays an indispensable role in guiding thalamocortical axons innervating the developing cortex (López-Bendito, 2018). Cortical interneurons are generated in the ventral telencephalon, tangentially migrating from their germinal centers into the cortex (Lim et al., 2018; Silva et al., 2019). In humans, an expanded germinal zone overlying the VZ/SVZ (the outer SVZ, oSVZ) harbors outer radial glia (oRG), which generate cortical neurons and are thought to contribute to the evolutionary expansion of the human neocortex (Fietz et al., 2010; Hansen et al., 2010). Not depicted are less abundant cell types with important roles in cortical development, including microglia, endothelial cells and pericytes.

2.1 – Cortical NSPC proliferation

2.1.1 Spontaneous calcium elevations

Radial glial cells (RGCs) and intermediate progenitor cells (IPCs) comprise the proliferative NSPC compartment giving rise to cortical excitatory neurons and astrocytes. Residing adjacent to the ventricles, these cells are exposed to environmental stimuli impinging on calcium signaling during development, including growth factors and electrical activity (Dehay & Kennedy, 2007; Fame & Lehtinen, 2020). Cortical NSPCs express ion channels, pumps and receptors that generate unique calcium dynamics to contribute to developmental cellular behaviors (**Figure 2.2**). Different patterns of spontaneous calcium elevations are observed in the ventricular zone (VZ): slow rises are contained to individual cells, while coordinated transients (calcium waves) propagate across

gap junction-coupled, mitotically-active RGCs (Bittman et al., 1997; Lo Turco & Kriegstein, 1991; Owens et al., 2000; Weissman et al., 2004).

Spontaneous calcium rises are dependent on internal calcium stores, as they persist in the absence of extracellular calcium but are eliminated upon ER calcium depletion (Owens & Kriegstein, 1998). VGCC activation, neurotransmitter signaling and depolarization are not necessary to promote spontaneous rises (Owens & Kriegstein, 1998; Weissman et al., 2004). Instead, initiation of these calcium transients requires purinergic signaling via metabotropic P2Y1 ATP receptors (P2Y1Rs) (X. Liu et al., 2008; Malmersjö et al., 2013; Owens et al., 2000; Owens & Kriegstein, 1998; Weissman et al., 2004), and NSPCs have been identified as a source of ATP eliciting pro-proliferative calcium responses (J. H.-C. Lin et al., 2007). Calcium waves are activated by extracellular ATP in a temporally regulated fashion, occurring robustly at the peak of neurogenesis and propagating across dynamically coupled RGCs via connexin hemichannels at specific cell cycle stages (Bittman et al., 1997; Owens & Kriegstein, 1998; Weissman et al., 2004). Abrogating these waves by antagonizing P2Y1Rs or inhibiting gap junctions significantly reduces proliferation and promotes differentiation (J. H.-C. Lin et al., 2007; Malmersjö et al., 2013; Weissman et al., 2004). Single cell RNA sequencing (scRNAseq) and calcium imaging studies demonstrate that P2Y1Rs are highly expressed in ventricular RGCs and IPCs of the rodent and human cortex and that P2Y1R agonists induce calcium rises in these cells (S. Mayer et al., 2019). P2Y1Rs are downregulated in neurons and, intriguingly, in human outer radial glia (oRG), a neural stem cell population abundant in humans that may contribute to evolutionary expansion of the neocortex, pointing to a conserved role for ATP-dependent calcium signaling in ventricular RGCs and IPCs (X. Liu et al., 2008; S. Mayer et al., 2019).

2.1.2 Links between calcium and growth factor signaling

Growth factors are important cell cycle regulators that induce calcium elevations in proliferative NSPCs (Dehay & Kennedy, 2007). High concentrations of basic fibroblast growth factor (bFGF), for example, elicit robust cytoplasmic calcium rises in the apical end foot and cell body of RGCs, propagating in a sustained manner through the RGC fiber (Rash et al., 2016). bFGF can promote proliferation in concert with epidermal growth factor (EGF) (Tropepe et al., 1999), which stimulates depletion of ER calcium stores to activate SOCE and induce NFAT-dependent transcription in ganglionic eminence (GE)-derived ventral progenitors and adult subventricular zone (SVZ) cells (Somasundaram et al., 2014). NFATs have been linked to cell cycle regulation (Carvalho et al., 2007; Teixeira et al., 2016) and progenitor proliferation in the neural tube and postnatal neural cultures (Huang et al., 2011; Serrano-Pérez et al., 2015). In GE progenitors, inhibiting SOCE using genetic and pharmacological approaches decreases proliferation (Somasundaram et al., 2014). While the function and upstream activators of SOCE in cortical NSPCs remains unknown, spontaneous calcium waves in the VZ are partially mediated by intracellular calcium stores and IP3-dependent calcium release (Weissman et al., 2004). Application of the IP3 receptor antagonist 2-aminoethoxydiphenyl borate, at doses that inhibit SOCE, reduces the amplitude and duration of calcium transients in cortical RGCs (Rash et al., 2016), pointing to potential roles for SOCE in proliferative NSPCs.

2.1.3 Neurotransmitter signaling and calcium

Neurotransmitter signaling also influences cortical NSPC calcium elevations to regulate cell division. In the embryonic brain, upon binding to $GABA_AR$s, the neurotransmitter GABA depolarizes immature cells (Ben-Ari et al., 2007). $GABA_AR$-dependent depolarization results from

unopposed developmental activity of the Na^+–K^+–$2Cl^-$ transporter NKCC1, which yields elevated chloride concentrations in embryonic neural cells (Ben-Ari et al., 2007; Owens et al., 1996, 1999). The postnatal emergence of the K^+-Cl^- co-transporter KCC2 induces a developmental switch in GABA activity from depolarizing to hyperpolarizing (Ben-Ari et al., 2007). This postnatal maturation of inhibition is regulated by activity of the largely transient subplate (SP) neuron population (Kanold & Shatz, 2006), by GABA (Ganguly et al., 2001), and by growth factor signaling (Rivera et al., 2004). The role of neural activity in the developmental regulation of intracellular chloride and transition to GABA inhibition is supported by studies demonstrating that manipulating excitatory input or sensory experience modulates KCC2 expression (S. He et al., 2010; Kanold & Shatz, 2006; Sernagor et al., 2003). Activity and cytosolic calcium also alter chloride gradients in immature hippocampal neurons (Fiumelli et al., 2005), and activity-dependent neurotrophins modulate KCC2 expression and GABAergic inhibition (Aguado et al., 2003; Ludwig et al., 2011).

Embryonic cortical NSPCs express functional $GABA_A$ receptors (LoTurco et al., 1995; S. Mayer et al., 2019; Owens et al., 1996), and GABA-induced depolarization elicits calcium transients in NSPCs through VGCC activation (LoTurco et al., 1995; S. Mayer et al., 2019; Owens et al., 1996, 1999; Panagiotakos et al., 2019). GABA depolarization inhibits DNA synthesis, decreasing cortical NSPC proliferation (Antonopoulos et al., 1997; LoTurco et al., 1995). This effect is rescued by application of chloride transport blockers, suggesting that the depolarizing activity of GABA underlies its ability to suppress NSPC proliferation (LoTurco et al., 1995). Importantly, separately dissecting the effects of GABA in the VZ and SVZ revealed that GABA inhibits SVZ progenitor proliferation but has pro-proliferative effects in the VZ, shortening the cell cycle to promote mitotic re-entry (Haydar et al., 2000). These data suggest that GABA exerts

cell type-specific effects on RGCs and IPCs, but how they are transduced to influence calcium in each cell type remains unknown. Understanding how developmental GABAergic activity is linked to the electrical properties of progenitor types, which can change across developmental time (Vitali et al., 2018), represents an exciting avenue for future studies.

Likewise, the excitatory neurotransmitter glutamate depolarizes NSPCs, promoting calcium rises that control proliferation. While NMDARs are expressed in the developing brain (Henson et al., 2008; LoTurco et al., 1991; Monyer et al., 1994), a confluence of data points to NMDARs playing an outsized role in post-mitotic neurons compared to NSPCs (Behar et al., 1999; LoTurco et al., 1991, 1995; Maric et al., 2000; S. Mayer et al., 2019). In the rat VZ, NMDA does not elicit currents in NSPCs, while in human RGCs and oRG, NMDA elicits magnesium-insensitive currents at resting membrane potentials (LoTurco et al., 1991, 1995; Maric et al., 2000; S. Mayer et al., 2019). Calcium imaging of developing human cortical cells identified a small fraction of VZ cells responding to NMDA, but scRNAseq reveals that these cells are predominantly excitatory neurons and mostly absent from NSPC clusters (Mayer et al., 2019).

In contrast, ionotropic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and □hospho (KA) receptors are expressed in VZ/SVZ cells (Haydar et al., 2000; LoTurco et al., 1995; Maric et al., 2000). Four main subunits, GluA1-4, compose heteromeric AMPA receptors (AMPARs) (Traynelis et al., 2010), and the presence of GluA2 renders AMPARs calcium impermeable (Sommer et al., 1991). Intriguingly, GluA2 abundance increases postnatally, suggesting that embryonic neural cells are more likely to express calcium-permeable AMPARs (Behm & Öhman, 2016; Kumar et al., 2002). In line with this, AMPA induces calcium elevations in isolated NSPCs completing their final division (Maric et al., 2000), and AMPA/KA antagonists block calcium elevations elicited by glutamate in rat cortical VZ cells (LoTurco et al., 1995).

AMPAR stimulation in isolated human fetal cortical NSPCs also promotes differentiation (S. Mayer et al., 2019; Whitney et al., 2008). Consistent with these findings, glutamate and KA (but not NMDA) significantly inhibit DNA synthesis in VZ cells (LoTurco et al., 1995). Once again, separating RGCs from IPCs reveals a more complex picture: glutamate increases proliferation in the VZ and decreases SVZ proliferation, underscoring differential responses of distinct progenitor populations to neurotransmitter signaling (Haydar et al., 2000). It will be important to dissect these responses across development, particularly in light of data demonstrating progressive RGC hyperpolarization during cortical neurogenesis as a key regulator of RGC output (Vitali et al., 2018). Notably, among the channels upregulated to promote this developmental change in RGC membrane potential are several calcium-activated K^+ channels (Vitali et al., 2018). While dynamic changes in K^+ channel expression and membrane potential have been linked to the control of proliferation (Blackiston et al., 2009), how such changes influence NSPC calcium signaling remains unclear.

The neurotransmitters serotonin (5-HT) and acetylcholine (Ach) also induce NSPC calcium transients. Maternally-derived 5-HT is reported to regulate proliferation in the developing brain (Côté et al., 2007); however, stimulation of 5-HT receptors HTR2A and HTR2C was shown to promote rat cortical progenitor survival without affecting proliferation (Dooley et al., 1997). HTR2A is highly expressed in human cortical germinal zones, and the HTR2A agonist TCB-2 induces robust calcium rises in human NSPCs (S. Mayer et al., 2019). HTR2A inhibition alters fiber length in proliferating human RGCs but does not impact division, positing a human-specific role for serotonergic signaling in maintaining RGC structural integrity during proliferation (S. Mayer et al., 2019). In contrast, muscarine and Ach stimulate calcium influx in cortical NSPCs via muscarinic Ach receptor (mAchR) activation, and mAchR antagonists and calcium chelators

attenuate NSPC proliferation (Atluri et al., 2001; W. Ma et al., 2000). In GE-derived progenitors, Ach, like EGF, stimulates SOCE via mAchRs, and abrogating SOCE reduces proliferation, pointing to potential links between cholinergic activity, SOCE and NSPC divisions (Somasundaram et al., 2014).

It is possible that agonist-induced SOCE and depolarization-induced calcium entry represent mechanisms that antagonistically regulate NSPC proliferation. Understanding the coordination of different modes of calcium entry and how their interplay impacts proliferative NSPCs is thus essential. Moreover, as calcium influx via VGCCs and SOCE can contribute to calcium oscillations, it will be critical to determine how cellular responses are encoded in patterns of calcium transients and how these dynamics enable signaling specificity in NSPCs.

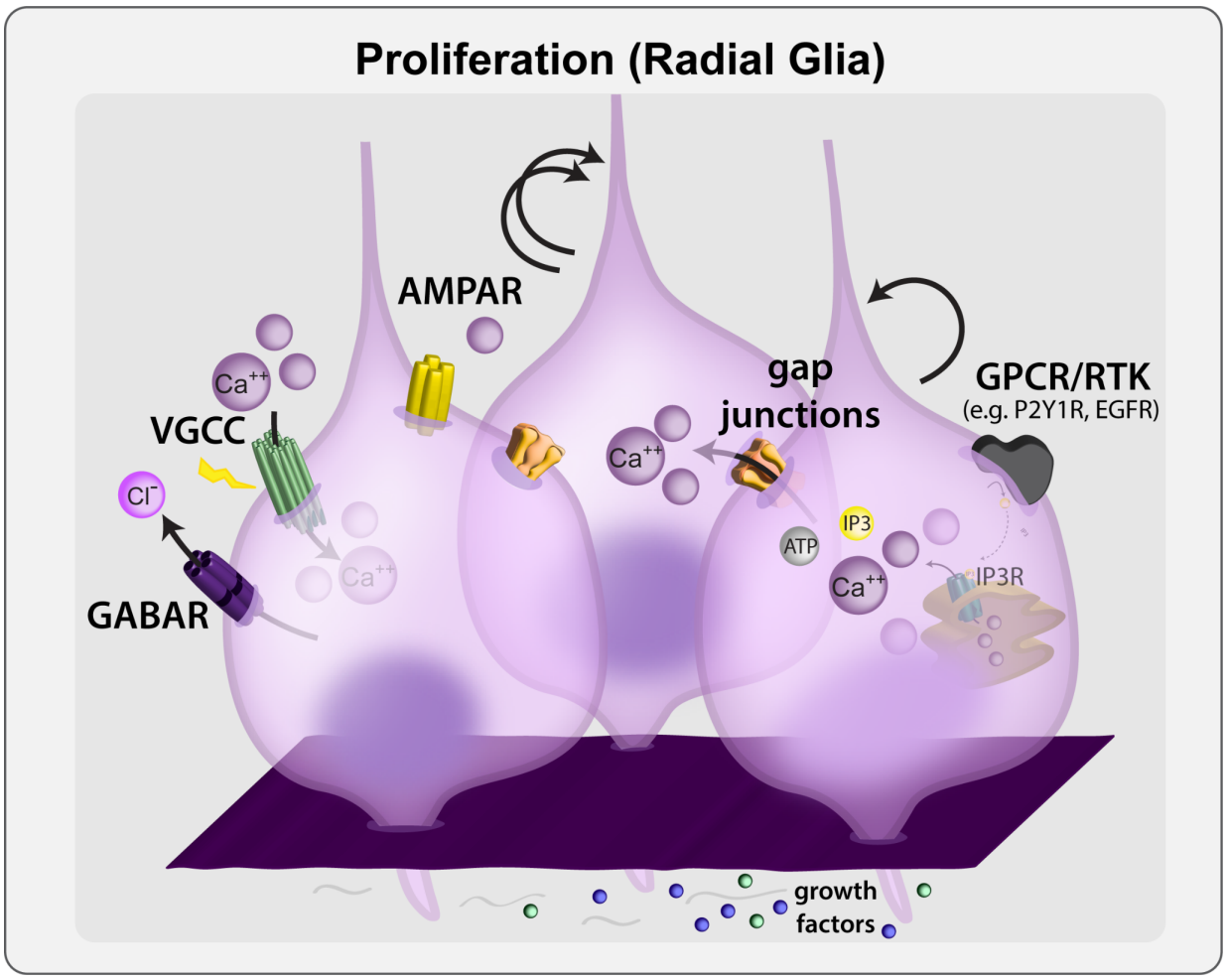

Figure 2.2 – Calcium-dependent regulation of proliferation during corticogenesis
Calcium imaging and electrophysiological recordings performed on embryonic rodent cortical slice cultures demonstrate that NSPCs (radial glia) in the ventricular zone (VZ) exhibit spontaneous and induced calcium rises. Purinergic signaling through metabotropic P2Y1 receptors initiates calcium transients that propagate across VZ cells. These calcium waves, which modulate proliferation, require gap junctions and IP3-mediated calcium release. RGC primary cilia protrude into the ventricles, where they are exposed to diffusible growth factors in the CSF that initiate calcium rises and also influence RGC division. Finally, depolarization mediated by GABA and glutamate acting on GABARs and AMPARs, respectively, controls proliferation by inducing calcium rises through VGCCs.

2.2 – Calcium-dependent cellular motility

2.2.1 NSPC motility

Proliferative RGCs undergo interkinetic nuclear migration (INM) – dynamic somatic movements in phase with the cell cycle (Taverna et al., 2014). During G1, RGC somata move away from the ventricle to complete S-phase, whereas they move apically during G2, initiating mitosis at the ventricular wall. Pharmacological inhibition of connexin hemichannels suppresses RGC calcium waves (X. Liu et al., 2010; Weissman et al., 2004) and significantly attenuates INM (X. Liu et al., 2010). Chelating intracellular calcium also reduces INM distance and speed (X. Liu et al., 2010), suggesting that calcium propagating through coupled RGCs plays a role in dynamic structural changes associated with RGC motility.

IPCs do not exhibit INM, instead delaminating from the VZ and moving into the SVZ along RGC fibers (Noctor et al., 2004; Taverna et al., 2014). Pharmacological inhibition of purinergic signaling or P2Y receptor knockdown reduces calcium transient frequency in proliferative IPCs, preventing their migration into the SVZ (X. Liu et al., 2008). Thus, ATP-mediated calcium signaling is not only necessary for NSPC proliferation, but also for dynamic progenitor movements that shape developing cortical cytoarchitecture (X. Liu et al., 2008).

2.2.2 Spontaneous calcium rises in postmitotic migratory cells

Spontaneous calcium rises have been linked to the radial migration of excitatory neuroblasts (**Figure 2.3**). Newborn neuroblasts exiting the VZ exhibit the highest frequency of somatic, bursting calcium transients in the developing cortex (Rash et al., 2016). It has also been postulated that calcium transients in RGC fibers, which act as a scaffold for radial migration, signal to neuroblasts to influence their calcium dynamics and migratory behavior (Rash et al., 2016). It

remains unclear, however, how calcium dynamics in RGCs and neuroblasts intersect, and what the functional contribution of bursting transients in migratory neuroblasts is to radial migration.

Unlike excitatory neurons, inhibitory cortical interneurons are generated in subpallial structures, including the medial and caudal ganglionic eminences (MGE and CGE, respectively), undertaking a saltatory tangential migration to reach the cortex (Buchsbaum & Cappello, 2019; Contractor et al., 2021; Lim et al., 2018; Silva et al., 2019). Within the cortex, interneurons undergo both tangential and radial migration to their appropriate laminar destination (Silva et al., 2019; Tanaka et al., 2006). Migratory interneurons exhibit spontaneous calcium transients characterized by oscillatory bursts or individual spikes (Bortone & Polleux, 2009; Martini & Valdeolmillos, 2010). Chelating intracellular calcium or pharmacologically blocking VGCC activity significantly disrupts cytoskeletal dynamics and impairs interneuron migration (Bortone & Polleux, 2009; Martini & Valdeolmillos, 2010). Caffeine-induced ER calcium release also promotes cytoskeletal changes to stimulate interneuron motility (Martini & Valdeolmillos, 2010). Interestingly, SOCE is attenuated in migratory neuroblasts isolated from the GE (Somasundaram et al., 2014), again raising the question of how different modes of calcium entry intersect in developing neurons to coordinate migration.

2.2.3 Agonist-induced calcium rises in migratory neuroblasts

Tyrosine receptor kinase B (TrkB), canonically bound by brain derived neurotrophic factor (BDNF), has been linked to embryonic cortical neuroblast migration (Bartkowska et al., 2007; Behar et al., 1997) (**Figure 2.3**). BDNF stimulates calcium transients and increases cortical neuroblast migration, which is blocked by application of Trk inhibitors or calcium chelators (Behar et al., 1997). These data suggest that TrkB activation promotes migration partly by inducing

downstream calcium signaling, although it should be noted that EGF robustly transactivates TrkB *in vivo* to stimulate cortical neuroblast migration (Puehringer et al., 2013).

TrkB is also involved in tangential interneuron migration (Contractor et al., 2021; Silva et al., 2019). BDNF and neurotrophin-4 (NT4) activate interneuron TrkB receptors, and application of Trk inhibitors reduces the fraction of MGE-derived cells migrating into the cortex. In mice lacking *Trkb*, the number of Calbindin-positive interneurons that reach the cortex is attenuated (Polleux et al., 2002). In contrast to TrkB-mediated activation of PLCγ and MAP kinase in excitatory neuroblasts, BDNF activates PI3-kinase signaling in interneurons to control tangential migration (Polleux et al., 2002). Intriguingly, BDNF- and NT4-dependent TrkB activation has been implicated in KCC2 regulation, positing a link between growth factor signaling and excitability in developing neurons (Rivera et al., 2004).

2.2.4 Neurotransmitter-induced calcium signaling in migrating neuroblasts

Intracellular calcium modulation via GABAR-mediated signaling also impacts radially migrating cortical neurons. In excitatory neuroblasts, GABA induces calcium transients and stimulates migration (**Figure 2.3**) (Behar et al., 1996). BAPTA blocks these effects, suggesting that calcium transduces GABA stimulation into migratory behavior (Behar et al., 1996). Indeed, $GABA_A$ and $GABA_B$ receptors are functionally expressed in migratory cortical neurons (López-Bendito et al., 2002; Owens et al., 1996, 1999). Pharmacological studies in embryonic rat cortical slices indicate that activation of different GABARs plays distinct region-specific roles across the cerebral wall (Behar et al., 2000). Similar observations have been replicated *in vivo*, where local application of $GABA_AR$ antagonists or chronic administration of desensitizing levels of $GABA_AR$ agonists attenuate spontaneous calcium transients, resulting in cortical heterotopias (N. Heck et al., 2007).

Notably, while GABA has been reproducibly implicated in migration, mice lacking *Gad65* and *Gad67* do not display major cortical malformations (F. Ji et al., 1999), and radial migration is not significantly altered in *Gad67* knockout mice (Furukawa et al., 2014). Other endogenous activators of $GABA_A$Rs (e.g., taurine) have thus been proposed to control neuronal migration (Furukawa et al., 2014).

Tangentially migrating cortical interneurons also display GABAR-mediated calcium transients (Soria & Valdeolmillos, 2002). As immature interneurons migrate from the MGE, GABA exerts a depolarizing effect via $GABA_A$Rs (Bortone & Polleux, 2009; Cuzon et al., 2006), and antagonizing $GABA_A$Rs results in accumulation of migrating interneurons at the pallial-subpallial boundary (Cuzon et al., 2006). GABA depolarization activates VGCCs to promote calcium influx, acting as a pro-migratory signal, presumably via calcium-dependent pathways to the cytoskeleton (Bortone & Polleux, 2009). During the first postnatal week in mice, KCC2 upregulation renders GABA hyperpolarizing, leading to decreased calcium transients and reduced interneuron motility to terminate migration (Bortone & Polleux, 2009). Suppressing activity of CGE-derived interneurons at distinct developmental stages does not affect tangential migration but reveals essential contributions for activity-dependent signaling in the radial migration of specific subpopulations into their final laminar positions in the CP (De Marco García et al., 2011).

Glycine receptor (GlyR) activation by endogenous ligands (e.g., glycine, taurine) is also depolarizing in cortical neuroblasts, eliciting calcium rises that may modulate migration (Avila et al., 2013; Flint et al., 1998; Yoshida et al., 2004). While cortices from mice lacking the developmentally enriched α2 GlyR display no gross morphological defects (Young-Pearse et al., 2006), genetic deletion of these receptors results in impaired interneuron migration (Avila et al., 2013). In migratory cortical interneurons, extrasynaptic glycine acting on α2 GlyRs stimulates

dynamic calcium fluctuations via VGCCs, promoting motility through calcium-dependent tuning of actomyosin contractions (Avila et al., 2013). In excitatory neuroblasts, the role of GlyRs is less clear (Furukawa et al., 2014), though GlyR activation in organotypic slice cultures, in the presence of glycine uptake inhibitors, impedes radial migration (Nimmervoll et al., 2011).

Seminal experiments in cerebellar granule cells first demonstrated calcium-dependent roles for glutamate in neuroblast migration (Komuro & Rakic, 1993). In the developing cerebral cortex, glutamate released by post-mitotic neurons induces calcium transients in radially migrating neuroblasts, modulating their motility primarily through NMDARs (**Figure 2.2**) (Behar et al., 1999; Hirai et al., 1999). NMDARs are heteromers, the majority consisting of two GluN1 and two GluN2 subunits. While GluN1 subunits are expressed before and after birth in the cortex, GluN2 subunit expression is dynamically regulated during development, resulting in NMDARs with markedly different physiological properties. Compared to those containing GluN2B subunits, GluN2A-containing NMDARs have faster deactivation kinetics, higher open probabilities, and lower sensitivity to agonists. While both NMDAR subtypes display similar calcium permeability, their unique gating properties shape the dynamics of their contribution to calcium influx to influence downstream signaling (Erreger et al., 2005; Paoletti et al., 2013; Sheng et al., 1994; Wyllie et al., 2013). In humans, *GRIN2B,* encoding GluN2B, is highly expressed in postmitotic embryonic neurons, whereas *GRIN2A*, encoding GluN2A, is expressed in embryonic RGCs and neurons after birth (S. Mayer et al., 2019). This developmental subunit switch, which occurs in early postnatal life in the rodent and can be regulated by neural activity (Yashiro & Philpot, 2008), results in enriched GluN2B abundance during the peak of neuroblast migration. Consequently, shRNA-dependent GluN2B and GluN1 knockdown in embryonic rodent cortices delays neuronal migration, whereas manipulating GluN2A does not affect neuroblast motility (Jiang et al., 2015).

Pharmacological NMDAR inhibition or calcium chelators in the presence of NMDA also abrogate neuroblast migration (Behar et al., 1999; Hirai et al., 1999; Reiprich et al., 2005; Yuryev et al., 2018). More recently, transient glutamatergic transmission from SP neurons onto excitatory neuroblasts was shown to regulate neuroblast migration in an NMDAR-dependent manner (Ohtaka-Maruyama et al., 2018). While these studies suggest that NMDARs are essential for radial migration, genetic inactivation of *Grin1*, encoding GluN1, reveals no major deficits in neuronal distribution (Messersmith et al., 1997; Iwasato et al., 2000). This may result from mechanisms compensating for long-term GluN1 loss of function (Luhmann et al., 2015; Medvedeva & Pierani, 2020). In tangentially migrating cortical interneurons, NMDA and Kainate also induce calcium transients (Soria & Valdeolmillos, 2002), and activation of NMDARs or AMPARs positively regulates interneuron motility in a VGCC-dependent manner (Bortone & Polleux, 2009). Within the IZ, tangentially migrating interneurons continue to express calcium-permeable AMPARs (Métin et al., 2000), and in CGE-derived interneuron subtypes, 5-HT3A receptor activation induces calcium transients required for proper migration (Murthy et al., 2014).

2.2.5 VGCCs in neuroblast migration

A function for VGCCs in neuronal migration was initially described in cerebellar granule cells (Komuro & Rakic, 1992). In the embryonic cortex, L-type VGCCs are highly expressed in cortical neurons, and migrating upper layer neurons exhibit spontaneous L-type VGCC-mediated calcium transients (Kamijo et al., 2018). *In utero* $Ca_v1.2$ overexpression in excitatory neurons destined for upper cortical layers impairs radial migration, and a severe calcium influx-dependent migratory defect in this population results from electroporation of $Ca_v1.2$ channels bearing the TS mutation (Kamijo et al., 2018).

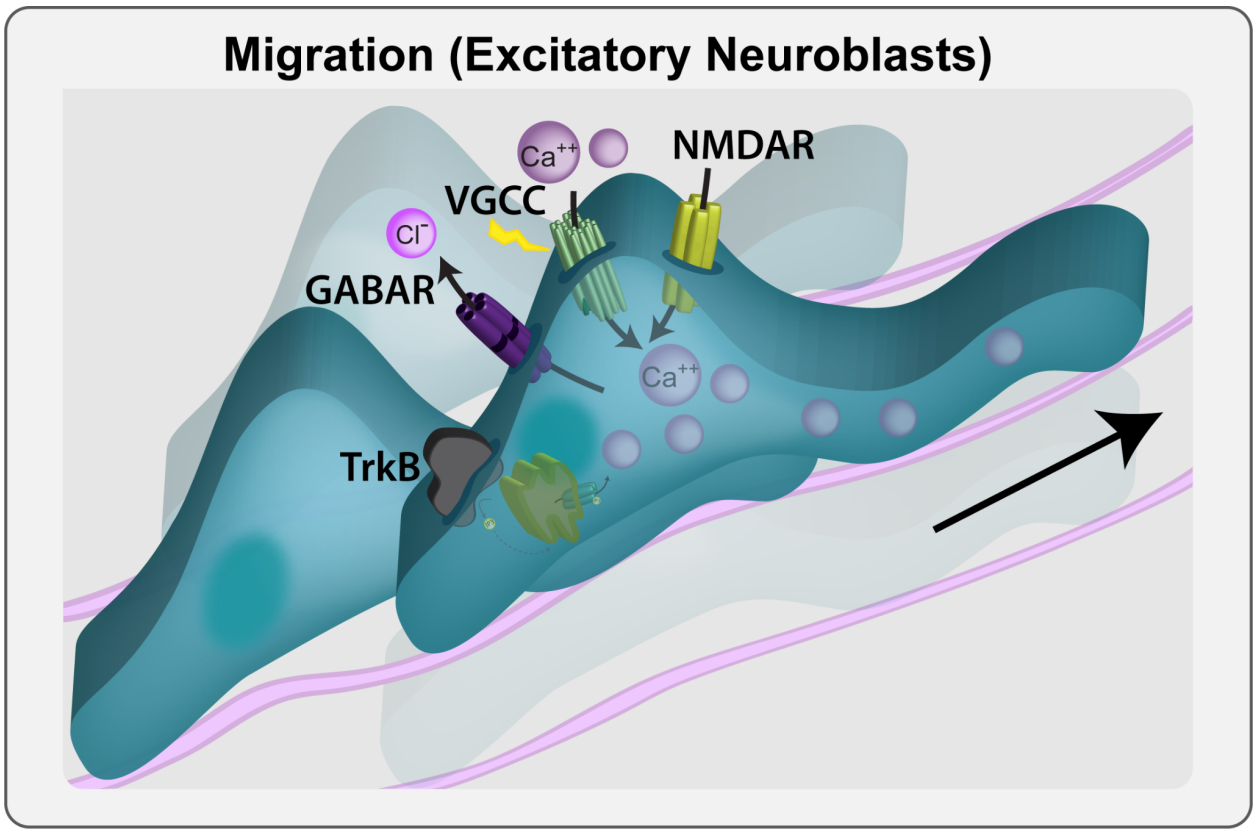

Figure 2.3 – Calcium and excitatory neuroblast migration
Excitatory neuroblasts undergo radial migration along RGC fibers to reach their final laminar destination. Migratory neuroblasts exiting the VZ display high frequency spontaneous calcium transients. As they enter the SVZ, neuroblasts adopt a multipolar morphology and exhibit low amplitude calcium events. Calcium transients during neuroblast migration are largely mediated by extracellular agonists like BDNF, which activates TrkB, by neurotransmitter receptors (e.g. NMDARs, GABARs), and by downstream activation of VGCCs. Glutamate influences radial migration by inducing calcium influx primarily through NMDARs. Depolarization via GABARs and calcium influx via VGCCs also control radial migration. Emerging evidence suggests that calcium transients propagating through RGC fibers may also contribute to the regulation of neuroblast migration into the CP.

L-type VGCCs, and to a lesser extent N-type VGCCs, are also essential for interneuron motility (Bortone & Polleux, 2009). Depolarization of immature interneurons by GABA or glutamate activates VGCCs to promote tangential migration into the cortex (Bortone & Polleux, 2009). Assembloid models using human iPSCs further support a role for $Ca_v1.2$ in human cortical

interneuron migration, demonstrating that inhibitory neurons from TS patients display abnormal migratory behaviors (Birey et al., 2017). Dissecting these migratory phenotypes demonstrates that VGCC-dependent calcium signaling impinges on distinct molecular networks to influence cellular motility (Birey et al., 2022).

2.3 – Calcium and programmed cell death

Developing cortical circuits are refined by precisely regulated apoptosis, which scales down NSPC, pyramidal cell and cortical interneuron populations. Approximately 12% of cortical pyramidal cells undergo apoptosis in the early postnatal period in rodents (Wong et al., 2018), and a substantial fraction of cortical interneurons also undergo postnatal cell death (Priya et al., 2018; Southwell et al., 2012). In both developing populations, a central regulator of apoptosis is neuronal activity and intracellular calcium signaling.

In vitro studies in the 1990s first implicated activity-dependent calcium signaling in cortical pyramidal neuron survival (Ghosh et al., 1994; Voigt et al., 1997). Stimulation of embryonic cortical cultures with potassium chloride (KCl) enhanced cell survival by promoting neurotrophin expression, an effect that was eliminated by chelating calcium or pharmacologically blocking L-type VGCCs (Ghosh et al., 1994). NMDARs contribute to developmental apoptosis of pyramidal cells during discrete temporal windows, as early postnatal NMDAR inhibition promotes cell death. This effect is rescued by concurrent VGCC activation, suggesting that VGCCs and NMDARs have overlapping roles in promoting calcium-dependent survival (Ghosh et al., 1994; N. Heck et al., 2008; Ikonomidou et al., 1999). The contribution of GABA to cortical neuron survival is again linked to its depolarizing or hyperpolarizing activity. Global NKCC1 inactivation reduces developmental cell death of transient layer 1 Cajal Retzius (CR) neurons (Blanquie,

Liebmann, et al., 2017), and hyperpolarization promotes subtype-specific survival of CR neurons (Riva et al., 2019). Inhibiting activity altogether in developing pyramidal cells significantly reduces their survival (Voigt et al., 1997), and regional *in vivo* variation in apoptosis is partly regulated by corresponding differences in endogenous activity (Blanquie, Yang, et al., 2017). *In vitro* evidence suggests, however, that it is not simply the presence or absence of activity that regulates apoptosis in developing cortical neurons; rather, distinct activity patterns may modulate survival, further supporting that information encoded in the frequency of calcium signals can drive changes in cell behavior (Golbs et al., 2011; Wong Fong Sang et al., 2021). Investigating how ion channel subunit composition and localization contributes to activity patterns *in vivo* and how these patterns activate specific calcium-dependent signaling pathways in developing cortical neurons to regulate survival is an essential next step.

Notably, the emergence of synchronous calcium transients within populations of cortical pyramidal and hippocampal neurons is positively correlated with survival (N. Heck et al., 2008; Murase et al., 2011; Voigt et al., 1997). Such synchronous transients in neuronal domains are partially mediated by gap junctions (Kandler & Katz, 1998; Yuste et al., 1992), and pharmacological gap junction inhibition preserves spontaneous asynchronous activity but increases neuronal apoptosis (N. Heck et al., 2008). In line with this, multiple calcium-dependent transcription factors have been linked to neuronal survival, including MEF2 (L. Liu et al., 2003; Mao et al., 1999) and NFATc4 (Benedito et al., 2005; Quadrato et al., 2012).

Increased activity also correlates with improved postnatal survival of cortical interneurons (Denaxa et al., 2018; Priya et al., 2018; Wong et al., 2018, 2022). Interneurons that die are less likely to participate in coordinated network events, displaying fewer synchronized calcium fluctuations *in vivo* compared to cells that survive (Duan et al., 2020; Wong et al., 2018).

Accordingly, artificially hyperpolarizing interneurons decreases their survival, whereas increasing their activity promotes survival (Denaxa et al., 2018; Priya et al., 2018). Interestingly, the contribution of activity-dependent signaling to interneuron survival, thought to be mediated at least partly through the calcium-sensitive CaN/NFAT pathway (Priya et al., 2018), is subtype-specific (Denaxa et al., 2018; Priya et al., 2018). The activity of maturing neuronal populations also non-cell autonomously regulates interneuron subtype survival to shape developing circuits (Wong et al., 2018, 2022). Glutamatergic signaling is required for activity-dependent survival of neurogliaform and basket cells, whereas bipolar cells rely on serotonin to modulate activity-dependent survival (Wong et al., 2022). It will be especially interesting to determine the calcium-dependent pathways that link electrical signals to the survival of specific neuronal subtypes.

2.4 – Differentiation

Calcium and electrical activity play central roles in regulating aspects of neuronal identity. We restrict our focus here to a brief discussion of calcium functions in early events underlying cortical neuron differentiation, namely the acquisition of fate determinants and initial elaboration of neurites. The involvement of activity-dependent signaling in later events, such as synaptogenesis, circuit integration, maturation and plasticity, have been extensively reviewed elsewhere (Antón-Bolaños et al., 2018; Greer & Greenberg, 2008; Molnár et al., 2020; Pan & Monje, 2020; Rosenberg & Spitzer, 2011).

Pioneering studies in *Xenopus* spinal neurons demonstrated that intracellular calcium elevations regulate distinct aspects of differentiation, including neurotransmitter specification, maturation of ionic conductances and synaptic development (Borodinsky & Spitzer, 2007; Gu & Spitzer, 1995). In the developing cortex, expression of neuronal fate determinants may be calcium-

regulated. Elevated calcium in differentiating human iPSC-derived TS cortical neurons biases neuronal production *in vitro* (Paşca et al., 2011). Moreover, *in vivo* gain and loss of function of the L-type VGCC $Ca_v1.2$ in differentiating NSPCs bidirectionally modulates the relative abundance of cells expressing markers of callosal or subcerebral projection neurons in a calcium-dependent manner (Panagiotakos et al., 2019). $GABA_AR$-dependent activation of L-type VGCCs also controls morphological differentiation of pyramidal cells (Y. Chen & Ghosh, 2005; McAllister et al., 1996; Redmond et al., 2002; Wayman et al., 2006), and premature KCC2 expression *in vivo* reveals that GABA depolarization is necessary for dendritic maturation of cortical neurons (Cancedda et al., 2007). Additionally, elevating neuronal activity and spontaneous calcium transient frequency in migratory neurons arrests migration and induces precocious dendritic branching (Bando et al., 2016). Consistent with this, thalamocortical afferent activity is indispensable for proper development of neuronal morphology (Callaway & Borrell, 2011; H. Li et al., 2013), as well as barrel column formation in mice (Antón-Bolaños et al., 2019; H. Li et al., 2013). Recently, a small molecule chemical screen in human iPSC-derived cortical neurons identified epigenetic modifiers and calcium signaling activators as enhancers of neuronal maturation, including neurite elaboration, further supporting that facilitating calcium-dependent gene expression can promote terminal differentiation of cortical neurons (Ciceri et al., 2022; Hergenreder et al., 2022).

Calcium-regulated transcription factors or transcriptional activators, including CREB (Redmond et al., 2002; Wayman et al., 2006), NEUROD (Gaudillière et al., 2004) and CREST (Aizawa et al., 2004), have also been implicated in dendrite development. In addition, a CREB-dependent microRNA (miR132) positively regulates cortical neuron neurite outgrowth (Vo et al., 2005). Callosal axon outgrowth in the developing cortex is impeded by suppressing neuronal

activity (Mizuno et al., 2007; Rodríguez-Tornos et al., 2016; Suárez et al., 2014; C.-L. Wang et al., 2007), and axonal pathfinding has been linked to intracellular calcium signaling involving kinases and phosphatases with distinct calcium sensitivities (Gomez & Zheng, 2006). How these calcium-dependent mechanisms cooperate to control axonal and dendritic elaboration in the developing cortex remains unclear. Intriguingly, a calcium-independent interaction between $Ca_V1.2$ and RhoA was shown to regulate dendritic morphogenesis (Krey et al., 2013), highlighting additional roles for VGCCs as anchors for large signaling complexes at the membrane.

Electrical activity and neurotransmitter signaling regulate cortical interneuron differentiation in a calcium-dependent manner. BDNF, in conjunction with depolarization, enhances dendritic branching and electrophysiological maturation of parvalbumin (PV)-expressing interneurons (Berghuis et al., 2004). Postnatal electrical activity mediated by ionotropic glutamate receptors is also required for morphological maturation of calretinin (CR)- and reelin (RE)-expressing cortical interneurons (De Marco García et al., 2011). The activity-regulated DNA binding protein SATB1, which is associated with postnatal survival of somatostatin (SST)-expressing MGE-derived interneurons, is necessary for their maturation and terminal differentiation (Close et al., 2012; Denaxa et al., 2012). KCl-induced SATB1 upregulation is dependent on both calcium influx through L-type VGCCs and GABAR activation (Denaxa et al., 2012). More recently, the calcium-dependent transcription factor MEF2C was found to be required for the differentiation of PV-expressing cortical interneurons (C. Mayer et al., 2018). Activity-dependent splicing regulators such as the ASD-relevant RNA binding protein RBFOX1 (**Table 1**), which promote neuronal differentiation of cortical NSPCs (X. Zhang et al., 2016), also regulate distinct aspects of PV- and SST-expressing interneuron maturation and connectivity (Wamsley et al., 2018). These data reveal that activity-dependent calcium signaling is essential for the

acquisition of molecular identity and morphology in developing pyramidal and interneuron populations.

2.5 – Calcium and gliogenesis

2.5.1 Cortical Astrogliogenesis

To ensure that neurogenesis proceeds faithfully during early cortical development, RGCs actively inhibit intrinsic mechanisms of astrogliogenesis, including transcription of astrocyte-specific genes (Miller & Gauthier, 2007). Extrinsic signals acting via neurotrophic factors later stimulate cortical RGCs to become gliogenic (Qian et al., 1997; Song & Ghosh, 2004), in part via JAK/STAT pathway activation (Bonni et al., 1997). G protein signaling initiated by pituitary adenylate cyclase-activating polypeptide (PACAP) and transduced via calcium has been identified as a complementary mechanism involved in the onset of astrogliogenesis (**Figure 2.4**) (Nishimoto et al., 2007; Vallejo & Vallejo, 2002). Activation of PAC1 receptors by PACAP induces cyclic AMP (cAMP) production, which promotes astrocyte differentiation (Cebolla et al., 2008; McManus et al., 1999; Vallejo & Vallejo, 2002). PACAP stimulation elicits gradual intracellular calcium rises in NSPCs, while cAMP antagonism eliminates these rises and blocks astrocyte differentiation (Cebolla et al., 2008). During astrogliogenesis, calcium binding to the downstream regulatory element antagonist modulator (DREAM) is required for expression of the astrocyte-specific *glial fibrillary acidic protein* (*Gfap*) gene (Cebolla et al., 2008). These data indicate that PACAP induces cAMP-dependent calcium entry, enabling calcium-dependent astrocyte-specific gene transcription (Cebolla et al., 2008; McManus et al., 1999; Vallejo & Vallejo, 2002). PACAP signaling in mice lacking *Dream* fails to induce astrocyte differentiation, but this can be rescued

by JAK/STAT activation, suggesting that calcium-regulated DREAM signaling works in parallel to JAK/STAT signaling during astrogliogenesis (Cebolla et al., 2008).

Neural activity and downstream calcium signaling is also implicated in the development and function of cortical oligodendrocyte precursor cells (OPCs) and microglia. Dissecting how activity-dependent calcium signaling facilitates communication between developing neurons and glia will enhance our understanding of how the cortex is built.

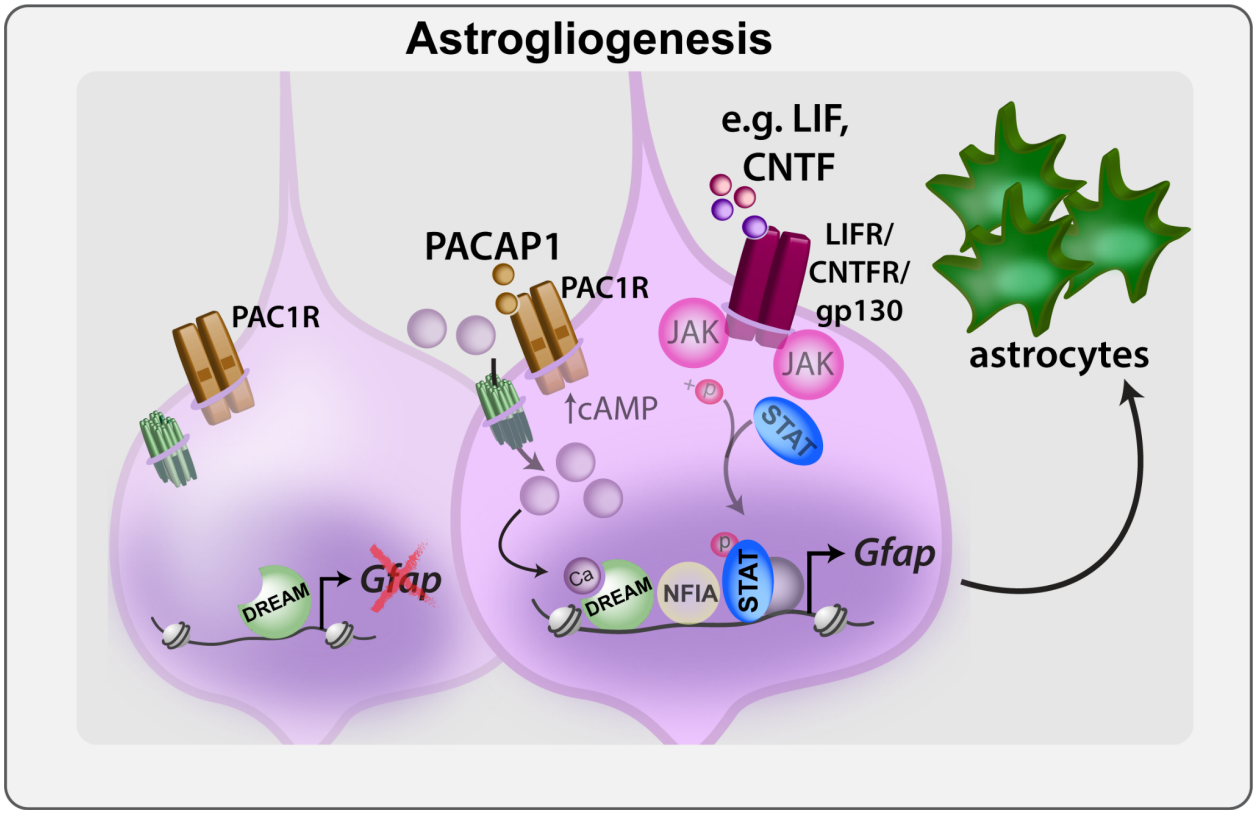

Figure 2.4 – Calcium and Astrogliogenesis

While microglia and embryonic oligodendrocytes originate largely outside of the cortex, RGCs (light purple) transition from making neurons to generating cortical astrocytes (green) during mid-to-late gestation in the rodent. This gliogenic switch begins at approximately embryonic day I17 in mice and continues postnatally. Activation of plasma membrane receptors (Glycoprotein 130 (gp130), Ciliary Neurotrophic Factor Receptor (CNTFR) and Leukemia Inhibitory Factor Receptor (LIFR)) regulates *Gfap* transcription and astroglial differentiation via JAK/STAT signaling. In parallel, Pituitary Adenylate Cyclase Activating Polypeptide (PACAP1)-mediated activation of its receptor, PAC1R, generates cAMP-dependent calcium elevations. Calcium binds DREAM to promote expression of astrocyte-specific genes and astrogliogenesis.

2.5.2 Embryonic glial cells originating outside the cortex

Embryonic oligodendrocyte precursor cells (OPCs) initially populate the cortex through two waves of migration from the ganglionic eminences (Kessaris et al., 2006). A third group of OPCs is generated in the postnatal cortex, replacing a subset of ventrally-derived OPCs (Kessaris et al., 2006). During these developmental windows, OPCs begin to express ion channels and neurotransmitter receptors that enable them to respond to activity-dependent signals (Bergles & Richardson, 2015; De Biase et al., 2010; Fulton et al., 2010; Spitzer et al., 2019). It was first demonstrated in the rodent optic nerve that inhibiting electrical activity reduces the number of mitotic OPCs (Barres & Raff, 1993). Pharmacological, electrophysiological and optogenetic manipulations in the cortex have since reinforced that neuronal activity regulates OPC proliferation and myelination (Demerens et al., 1996; Gary et al., 2012; Gibson et al., 2014; Mitew et al., 2018). The effects of electrical activity on OPCs are likely at least partly transduced via VGCCs, as $Ca_v1.2$ loss of function results in impaired OPC proliferation, axon-OPC interactions and myelination (Cheli et al., 2015, 2016). Live imaging of the developing zebrafish spinal cord also reveals that neuronal activity induces different patterns of calcium signals to regulate myelination (Baraban et al., 2017; Krasnow et al., 2018). What initiates these dynamics and how they influence genetic programs driving OPC differentiation and myelination remain avenues for future study. While OPCs differentially respond to activity-dependent signals in the spinal cord (Marisca et al., 2020), such OPC functional heterogeneity in the developing cortex remains poorly understood.

Microglia originate outside the CNS and migrate during embryonic development into the cortex (Ginhoux et al., 2010; Thion & Garel, 2017), where they play important roles in regulating progenitor abundance and sculpting developing circuits (Cunningham et al., 2013; Squarzoni et

al., 2014; Thion & Garel, 2020; Ueno et al., 2013). While various neurotransmitters, immune molecules and ligands for purinergic receptors induce microglial calcium rises *in vitro* (Umpierre & Wu, 2021), it remains less clear how calcium influences developmental functions of microglia in the cortex. Neuronal activity regulates microglial-mediated synaptic pruning (Schafer et al., 2012), and microglia sensitive to GABA preferentially remodel inhibitory synapses in the postnatal cortex (Favuzzi et al., 2021). In the developing zebrafish spinal cord, activity-dependent myelin phagocytosis by microglia is characterized by spontaneous calcium transients in microglial processes contacting myelin (Hughes & Appel, 2020). Future studies probing how activity and calcium facilitate neuron-glia communication during development will enable a framework for understanding how different cell types function in concert with each other to build the cortex.

2.6 – Calcium signaling deregulation in the context of disease

2.6.1 Reactivation of developmental calcium signaling mechanisms in glioma

In primary glioma specimens from adult human patients, stem-like glioma cells display molecular signatures reflecting NSPC identity (Bhaduri et al., 2020; Venteicher et al., 2017; R. Wang et al., 2020), and their morphology, behavior and lineage trajectory resembles that of embryonic RGCs (Bhaduri et al., 2020; Couturier et al., 2020; R. Wang et al., 2020). Similarities between cortical RGCs and stem-like glioma cells extend into calcium signaling dynamics, suggesting that calcium-regulated developmental mechanisms may be reused to promote glioma initiation and maintenance. For instance, patient-derived glioblastoma xenografts in mice display synchronous calcium transients (Venkataramani et al., 2019; Venkatesh et al., 2019) and, as in cortical RG, calcium and electrical activity modulates glioma cell proliferation (Urso et al., 2019; Venkatesh et

al., 2019; Y. Zhang et al., 2012). Adjacent glioma cells are connected via "microtubes" composed of gap junctions (Osswald et al., 2015), and calcium propagates across coupled cells in a manner reminiscent of calcium waves in the developing cortical VZ (Osswald et al., 2015; Venkataramani et al., 2019; Venkatesh et al., 2019). It is plausible that unraveling the calcium signaling mechanisms directing embryonic NSPC proliferation and migration will provide critical insights into glioma cell biology to identify potential therapeutic targets for the management of glioma invasion and progression.

2.6.2 – Implications for neurodegenerative diseases

Calcium acts as a node connecting environmental stimuli to intracellular signaling. As such, it is uniquely positioned to influence cellular behaviors that build the cortex and maintain its homeostasis throughout life. It is therefore unsurprising that calcium signaling deregulation has been implicated in neurodegenerative disorders, including Alzheimer's disease (AD) (Pchitskaya et al., 2018; Verkhratsky, 2019). In human iPSC models of AD and frontotemporal lobar degeneration-tauopathy, activity-dependent calcium elevations are abnormally high (Imamura et al., 2016; J. Park et al., 2018). Neurons and glia also display elevated basal calcium in AD mouse models (Pchitskaya et al., 2018; Verkhratsky, 2019), and genes related to increased cytosolic calcium are enriched in patients with heightened risk for sporadic AD (A. Heck et al., 2015). Intriguingly, calcium- and activity-dependent pathways altered in neurodevelopmental diseases are thought to be affected in AD (Ivashko-Pachima et al., 2021; Mencer et al., 2021). Key calcium signaling effectors like DYRK1A (**Table 1**) are deregulated both in neurodevelopmental disorders and cortices of sporadic AD patients (Ferrer et al., 2005). Consistent with this, aging and AD rodent models exhibit aberrant calcium-dependent CaN/NFAT signaling (Foster et al., 2001;

Norris et al., 2005), and blocking CaN/NFAT in AD models improves synaptic function, amyloid pathology, and astrogliosis (Furman et al., 2012).

Emerging studies also suggest that individuals with neurodevelopmental disorders such as ASD and Down syndrome (DS) have increased risk of developing AD and related dementias early in life (Lott & Head, 2019; Vivanti et al., 2021). While, in the case of DS, association with early-onset dementia is related to increased amyloid precursor protein (APP) resulting from trisomy of all or part of chromosome 21, multiple genetic and environmental factors likely contribute to increased AD susceptibility. Deregulation of electrical activity, which impinges on calcium, represents one possible contributing mechanism common to neurodevelopmental and neurodegenerative disorders. In DS and ASD mouse models, abnormalities in NKCC1/KCC2 expression promote persistent depolarizing activity of GABA, akin to the developing brain, in adult and postnatal hippocampal neurons (Deidda et al., 2015; Tyzio et al., 2014). Consequently, administration of the NKCC1 antagonist bumetanide in these models restores chloride gradients and rescues cognitive and behavioral abnormalities (Deidda et al., 2015; Tyzio et al., 2014). Intriguingly, bumetanide was recently shown to improve pathological and behavioral deficits in an AD rodent model (Taubes et al., 2021). furthermore, APP has been linked to the regulation of KCC2, GABAR and VGCC expression (M. Chen et al., 2017; Doshina et al., 2017; Yang et al., 2009), implicating it as a regulator of electrical activity and calcium homeostasis.

2.7 – Conclusions

As reviewed above, coordinated spatiotemporal regulation of calcium signaling directs cellular behaviors that underlie early cortical development, including aspects of NSPC function. Calcium is a key mediator of activity-dependent gene expression, serving as a hub linking

environmental cues to the cytoskeleton, metabolic pathways, and other biochemical cascades. Underscoring these critical developmental roles, genetic studies reveal that mutations in genes encoding calcium signaling modulators contribute to the pathophysiology of neurodevelopmental disorders. There remain, however, open questions centered around how activity and calcium-dependent processes are regulated at a cell type-specific level and how this regulation coordinates cellular behaviors during corticogenesis. In other cell and tissue types, properties of intracellular calcium dynamics have been linked to activation of specific downstream transcription factors. It is thus tempting to hypothesize that distinct cell type-specific patterns of electrical signals and calcium elevations in the embryonic cortex may subserve specific developmental roles. A natural corollary to this hypothesis is that disrupting calcium signaling and patterned electrical activity in specific cortical populations during development may promote neurodevelopmental disease.

Much remains to be elucidated about how calcium signaling is initiated and transduced in developing cortical cells. Titration of calcium signaling is not only achieved through dynamic regulation of ion channels and signaling proteins but also via coordination of extrinsic cues, including maternal hormones and metabolic regulation (Rash et al., 2018; Tyzio et al., 2006), which indirectly influence calcium homeostasis. How do extrinsic signals converge to shape calcium entry in different cortical cell populations? How do different modes of calcium entry work cooperatively to direct gene expression programs across cell types? How might intracellular calcium signals in one population influence dynamic interactions with other cell types? Do compartmentalized calcium signals that propagate across polarized cells, as reported in RGCs, influence local cellular processes (e.g., translation)? How do intraorganellar calcium stores contribute to calcium dynamics in developing cortical cells? Single cell, long read and spatial sequencing technologies, organoid modeling, subcellular calcium and voltage imaging

approaches, optogenetic and chemogenetic tools for manipulating and reading out neural activity, and advances in labeling and isolating developing cortical cell populations will allow us to interrogate functional consequences of electrical activity and calcium signaling with unprecedented cellular resolution.

Determining how disease-relevant mutations affect intracellular signaling and cell type-specific developmental behaviors across space and time (Panagiotakos & Pasca, 2022) will also enable therapeutic approaches for neurodevelopmental diseases targeting calcium and activity-dependent mechanisms. Of special interest is determining how early disruption of activity-dependent signaling might cascade into later developmental events to promote disease. Recent findings support the notion that neurodevelopmental mechanisms may be reactivated to promote adult and aging-related disease states, reinforcing the significance of exploring how calcium signals are normally regulated to coordinate the development of functional circuits. Looking ahead, it will be important to consider how changes at the organismal level (e.g., immune function, metabolism, gut-brain axis) may contribute to the regulation and misregulation of calcium in the embryonic and adult cortex to better understand normal development and the emergence of neurological disorders.

Chapter 3 – Investigating Roles of Store-Operated Calcium Entry in the Developing Cerebral Cortex

Chapter 3 is a manuscript in preparation.

Arjun McKinney A, Pedrozo V, Tong J, Launer S, Khan Y, Haveles C, Kriegstein AR, & Panagiotakos G. "Investigating the roles of Store-Operated Calcium Entry in the Developing Cerebral Cortex"

3.1 – Introduction

The embryonic cortical germinal zones consist of neural stem and progenitor cells (NSPCs) that proliferate, migrate and differentiate in a systematic fashion to produce all of the excitatory neurons and later, glial cells of the developing cortex. NSPC divisions are precisely regulated across developmental time by the convergence of environmental signals and intracellular genetic programs (Charron & Tessier-Lavigne, 2005; Chini & Hanganu-Opatz, 2021; Fernández et al., 2016; Florio & Huttner, 2014; Juric-Sekhar & Hevner, 2019; Taverna et al., 2014; Tiberi et al., 2012; D. D. Wang & Kriegstein, 2009). Calcium and calcium-dependent signaling represent a mechanism that translates extracellular stimuli into long-lasting intracellular biochemical signaling cascades in embryonic NSPCs to regulate their behavior and output.

Initial studies describing calcium elevations in the developing rodent cortex revealed various patterns of spontaneous calcium transients in embryonic ventricular zone NSPCs. Some spontaneous calcium rises were confined to individual cells, whereas coordinated calcium transients were observed to spread through groups of gap junction-coupled VZ cells (Owens &

Kriegstein, 1998; Weissman et al., 2004). These "waves" of calcium that propagate across clusters of cells in the VZ are important temporally-regulated mediators of NSPC proliferation (Weissman et al., 2004). Notably, depleting ER calcium stores significantly attenuated spontaneous cortical calcium waves, revealing the importance of intracellular stores in sustaining these patterned calcium elevations. Pharmacological inhibition of other mechanisms that contribute to calcium influx into the cytosol, including sodium channels, voltage gated calcium channels, GABA receptors, glutamate receptors and NMDA receptors, failed to eliminate calcium waves (Owens and Kriegstein 1998, Weissman et al., 2004), further underscoring the outsized role of ER calcium stores in mediating spontaneous NSPC calcium rises. Nevertheless, relatively little is known about the regulation and maintenance of intracellular calcium stores in the developing cortex. The functional significance of these ER stores, particularly with regards to how they regulate the developmental cellular behaviors of NSPCs of the embryonic cortex, also remains unknown.

Store operated calcium entry (SOCE) is a calcium signaling mechanism that is engaged to refill depleted ER calcium stores. ER store depletion typically follows activation of cell surface receptors that promote IP3 or Ryanodine mediated release of ER calcium stores. Residing in the ER membrane and containing an EF hand calcium-sensing domain that extends into the ER lumen, STIM calcium sensors are poised to detect when ER calcium levels are reduced below a certain threshold, at which point they oligomerize and interact with plasma membrane ORAI channels. This STIM-ORAI interaction initiates calcium influx through the ORAI channel pore, which has high specificity to calcium (for human ORAI1, this is likely due to a negatively charged glutamate residue at position 106 in its first transmembrane segment) (McNally et al., 2009; Prakriya et al., 2006). Upon entering the cytosol, calcium is then actively pumped into the ER to refill calcium stores. While SOCE is an indispensable route of calcium entry in many other tissues (Baba et al.,

2008; Davis et al., 2015; Feske, 2009; Gwack et al., 2008; Stiber et al., 2008; Vig et al., 2007), it has been largely understudied in the context of embryonic brain development. Intriguingly, a growing body of work is revealing that deficits in calcium signaling mechanisms contribute to neurodevelopmental disease. Of particular interest are risk genes that have been implicated in autism spectrum disorders (ASD) that encode proteins that handle calcium from ER stores (e.g., *ITPR1*, which encodes the inositol 1,4,5-trisphosphate receptor type 1 (IP3R1)) or channels that control calcium homeostasis (e.g., *ATP2B2*, which encodes the plasma membrane calcium ATPase, Plasma Membrane Ca2+ Transporting 2 (PMCA2)) (Carayol et al., 2011; De Rubeis et al., 2014; Iossifov et al., 2014; Schmunk et al., 2015, 2017; Takata et al., 2018; T. Wang et al., 2016). These genetic data point to a need for better understanding the mechanisms and molecular players that regulate calcium homeostasis, calcium signaling and intracellular calcium stores in the developing cerebral cortex.

Our work interrogates the regulation and function of SOCE in NSPCs of the developing mouse and human embryonic cortex. Using both calcium imaging and cell type-specific expression analysis, we show that *Orai1*, *Orai2* and *Stim2* are the primary mediators of SOCE. These gene products are dynamically expressed during cortical development, where they drive calcium elevations in response to pharmacological depletion of ER stores in NSPCs. We demonstrate that *Orai1* and *Orai2* are functionally required for SOCE in cortical NSPCs and that purinergic signaling can activate SOCE via *Orai1* and *Orai2* to initiate calcium influx. Our calcium imaging experiments further reveal that the amplitude of SOCE calcium responses is higher in proliferative NSPCs, and in accordance with this, our pharmacological experiments support the idea that SOCE is required for both mouse and human NSPC proliferation. In line with the idea of a role for SOCE in NSPC proliferation, we find that *in utero* over-expression of STIM2 isoforms that have opposing

effects on SOCE bidirectionally regulates cell cycle exit of cortical NSPCs. Altogether, our data to date suggests that SOCE is regulated dynamically in NSPCs to control their behavior.

3.2 – Results

3.2.1 SOCE is active in mouse and human primary cortical NSPCs

To determine whether SOCE is active in cells of the developing cortex, we performed calcium imaging of primary NSPC cultures that were isolated from embryonic day I13 mouse cortices. NSPCs were plated on an adherent substrate and transduced with a virus expressing the GcaMP7f genetically encoded calcium indicator (**Figure 3.2.1A-B)**. In our imaging paradigm, we depleted ER calcium stores using 1μM thapsigargin, an inhibitor of the SERCA pump, in 0mM calcium-containing media, which resulted in an increase in cytoplasmic calcium levels that represents efflux of calcium out of the ER. SOCE calcium rises were then recorded upon adding calcium back into the extracellular media (**Figure 3.2.1C**). Notably, these calcium rises were inhibited by concentrations of 2-aminoethoxydiphenyl borate (2-APB) that have been previously shown to inhibit SOCE (Prakriya & Lewis, 2001), supporting the notion that NPSCs display bona fide SOCE elevations. To interrogate whether SOCE is regulated dynamically during embryonic development, we next performed SOCE imaging of primary NSPCs isolated from E16 mouse cortices. E16 NSPCs were dissociated, plated and transduced with the GcaMP7f calcium indicator (**Figure 3.2.1D,E**). As in E13 NSPCs, E16 SOCE calcium rises were inhibited by 2-APB (**Figure 3.2.1F**). The amplitude of the SOCE rises was comparable in NSPCs cultured from these two developmental time points, though interestingly the area under the thapsigargin curve increased

from E13 and E16 (**Figure 3.2.2A-B**), suggesting that ER calcium store content may increase as development progresses.

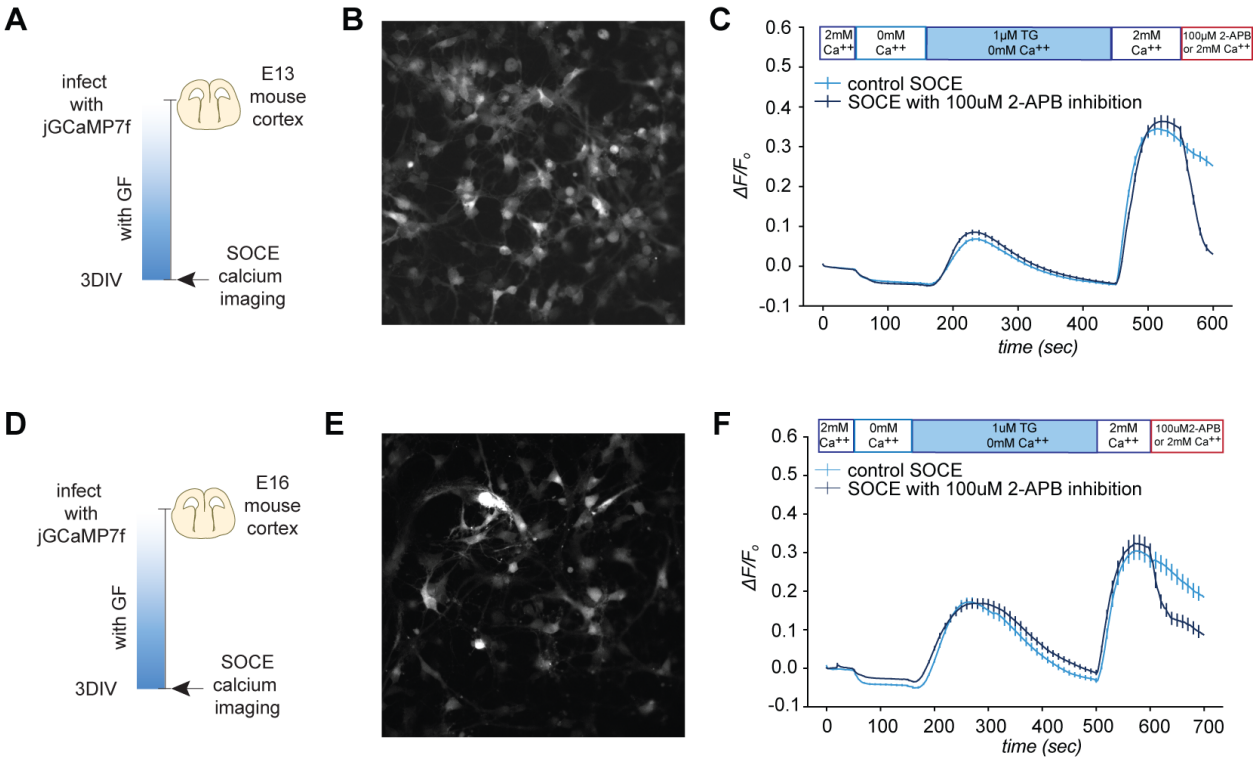

Figure 3.2.1 – SOCE is active in E13 and E16 mouse NSPCs
A) Schematic illustrates the experimental timeline of SOCE calcium imaging experiments performed on E13 embryonic mouse cortical cells maintained in media supplemented with growth factors for 3 days *in vitro* (DIV).
B) Representative field of view of GcaMP7f-expressing cells from imaging experiment in **C**.
C) Average GcaMP7f calcium imaging traces depict robust SOCE in E13 mouse embryonic NSPCs (mean ± SEM, n=504 cells represented in control trace (light blue), n=565 cells represented in 100μM 2-APB trace (dark blue)). The calcium imaging recording starts with the cells in 2mM calcium-containing media. The first calcium rise represents emptying of ER calcium stores using 1μM TG application in media containing 0mM calcium. To elicit SOCE, 2mM calcium is then reintroduced into the media. This second rise (representing SOCE) can be inhibited by application of 2-APB.
D) Schematic illustrates the experimental paradigm of SOCE calcium imaging experiments performed on E16 + 3DIV embryonic mouse cortical cells.
E) Representative field of GcaMP7f-expressing cells from imaging experiment shown in **F**.
F) Average GcaMP7f calcium imaging traces depict robust SOCE in E16 mouse embryonic NSPCs (mean ± SEM, n=357 cells represented in control trace (light blue), n=391 cells represented in 100uM 2-APB trace (dark blue)). As in **C**, the calcium imaging recording starts with the cells in 2mM calcium-containing media. The first calcium rise represents ER store depletion upon application of TG and the second calcium rise, representing SOCE, is elicited when

the cells are returned to 2mM calcium-containing media. Once again, this second rise can be inhibited by 2-APB.

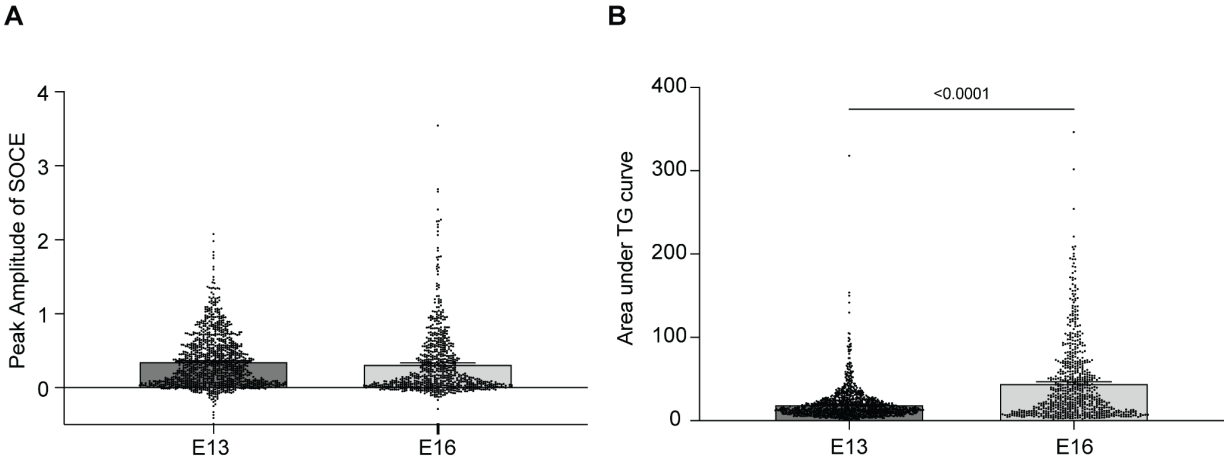

Figure 3.2.2 – Characteristics of SOCE recorded from E13 and E16 NSPCs
A) Summary data quantifying Peak SOCE Amplitude from calcium imaging traces of E13 and E16 NSPC cultures (bars present mean ± SEM; unpaired t-test)
B) Summary data describing the area under the TG curve from calcium imaging traces of E13 and E16 NSPC cultures (bars present mean ± SEM; unpaired t-test, $p < 0.0001$)

We complemented our SOCE recordings in primary mouse NSPCs with calcium imaging of primary NSPCs isolated from gestational week (GW) 19 human cortical tissue (**Figure 3.2.3 A**), in order to determine whether SOCE is also active in the developing human cortex. Germinal zones (comprising the VZ, iSVZ and oSVZ) were microdissected, dissociated and transduced with a lentivirus expressing GcaMP7f (**Figure 3.2.3B**). As with mouse NSPCs, human NSPCs display robust SOCE rises that can be inhibited by 100μM 2-APB (**Figure 3.2.3C**). Altogether, this data suggests that SOCE is functional in embryonic NSPCs of the mouse and human cortex.

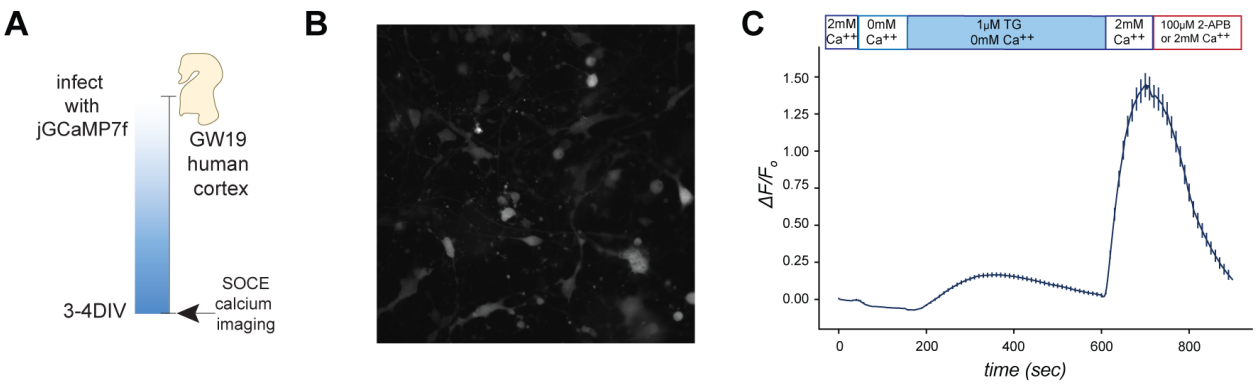

Figure 3.2.3 – SOCE is active in cells from human cortical germinal zones
A) Schematic illustrates the experimental timeline of SOCE calcium imaging experiments performed on GW19 human cortical cells dissociated from microdissected germinal zones.
B) Representative field of view of GcaMP7f-expressing cells from the calcium imaging experiment in **C**.
C) Average GcaMP7f calcium imaging trace depicts robust SOCE in human embryonic NSPCs (mean ± SEM; trace represents mean responses from 151 cells). As with recordings in mouse NSPCs, the calcium imaging recording begins with the cells in 2mM calcium-containing media. 1μM TG is applied to deplete ER stores and SOCE is then elicited by bathing the cells in 2mM calcium-containing media. The second calcium rise (representing SOCE) can be inhibited by 2-APB.

3.2.2 Orai1 and Orai2 mediate SOCE in cortical NSPCs

The calcium channels that mediate SOCE, also termed calcium release activated or CRAC channels, were first identified through genome wide RNA interference screens and genetic linkage mapping studies of individuals with CRAC channel deficits (Feske et al., 2006; Vig et al., 2006; S. L. Zhang et al., 2006). Since then, the identified plasma membrane channel, which has three different homologs, ORAI1-3, has been shown to mediate SOCE in nearly all cells in which it has been studied. To determine which ORAI homolog mediates SOCE in □hosphoping cortical cells, we first surveyed single cell sequencing datasets conducted on isolated cells from E12-E15 mouse cortices, which revealed that *Orai1* and *Orai2* are more highly enriched than *Orai3* (**Figure 3.2.4A-C**). Bulk RNA sequencing from the Brainspan dataset, which assayed human prefrontal cortical samples through fetal developmental and childhood, similarly revealed high expression of *ORAI1* and *ORAI2* in human cortical development in comparison to *ORAI3* (**Figure 3.2.5A-C**).

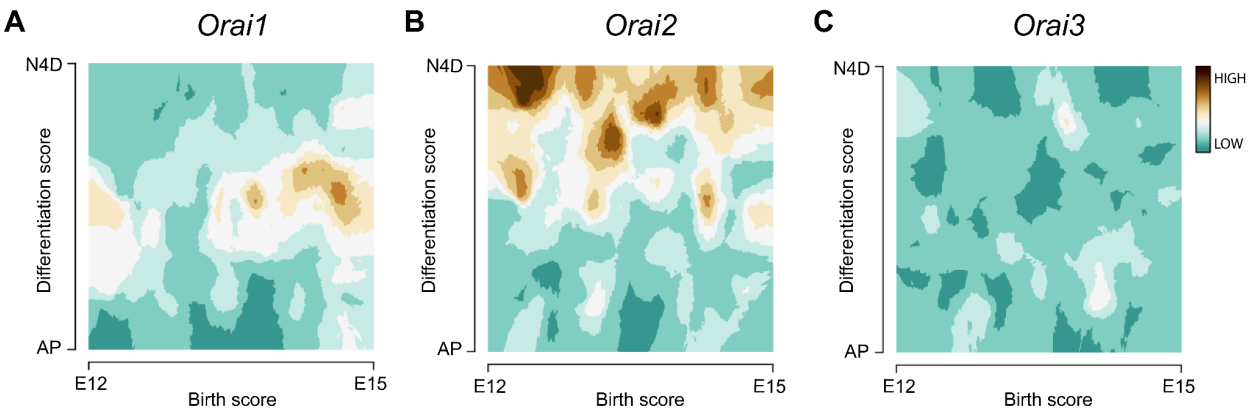

Figure 3.2.4 – *Orai1* and *Orai2* are highly expressed in the embryonic mouse cortex
A-C) *Orai1* (**A**), *Orai2* (**B**), and *Orai3* (**C**) expression from a single cell gene expression dataset from E12-E15 mouse cortices (Telley et al., 2019). In these plots, each cell has a defined birth score (x-axis, E12 to E15 corresponding to birthdate) and differentiation score (y-axis, corresponding to differentiation/maturation stage, ranging from apical progenitor (AP) to postmitotic cortical plate neuron (N4D)) (Telley et al., 2019). *Orai1* and *Orai2* are more highly expressed in the developing mouse cortex compared to *Orai3*, with *Orai2* expression particularly high in differentiating neurons.

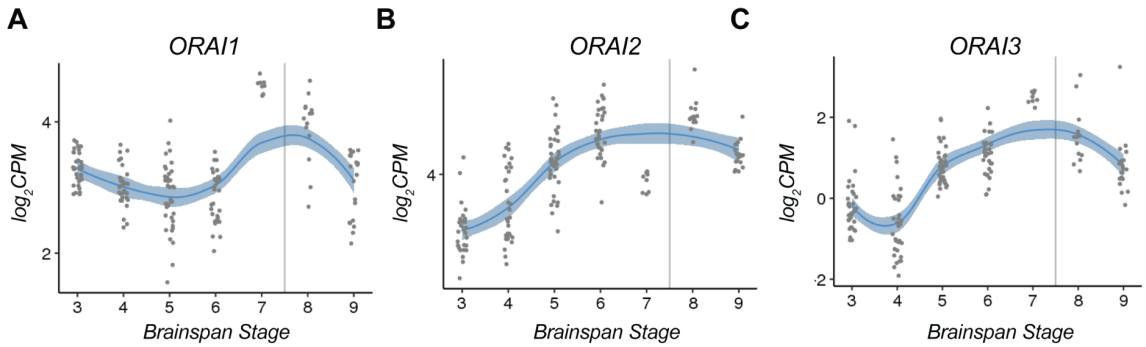

Figure 3.2.5 – *ORAI1 and ORAI2* are highly expressed in the embryonic human cortex

A-C) Expression levels in counts per million (CPM) of *ORAI1* (**A**) *ORAI2* (**B**), and *ORAI3* (**C**) from bulk RNA sequencing of 176 human prefrontal cortical samples ranging from Post Conception Week (PCW) 6 to 20 years of age (Brainspan dataset; stages on x-axis defined as: 3: 10PCW to 13PCW; 4: 13PCW to 16PCW; 5: 16PCW to 19PCW; 6: 19PCW to 24PCW; 7: 24PCW to 38PCW; 8: Birth to 6 months; 9: 6 months to 19 months (Gordon et al., 2021)).

To gain a better understanding of the cell type-specific expression of SOCE mediators in the developing cortex, we turned to Flashtag (FT), a carboxyfluorescein ester that labels mitotic radial glia and their progeny when injected into the ventricle, enabling the isolation of different

populations of embryonic cells (Govindan et al., 2018). We performed *in utero* intraventricular injections of FT at E13.5. We then used fluorescence activated cell sorting (FACS) to enrich for the 5-10% highest-expressing FT-positive cells at various time points, in order to isolate isochronic cohorts of cortical cells derived from mitotic radial glia lining the ventricles (**Figure 3.2.6**). In parallel to FACS, we collected FT-injected brains for immunofluorescence staining to validate the cell type identities of FT-labeled cells (**Figure 3.2.7A**). Cortices harvested at one hour after FT injection had FT-positive cells localized in the VZ that colocalize with the radial glia cell (RGC) marker SOX2 (**Figure 3.2.7B**). As expected, the most intensely FT-labeled cells reside along the ventricular wall, corresponding to mitotic RGCs. At 10 hours after injection, FT-positive cells were found predominantly in the SVZ and coexpressed the intermediate progenitor cell (IPC) marker TBR2 (**Figure 3.2.7C**), whereas immunostaining performed on cortices 24 hours after injection revealed two distinct populations: one "FT-high" migratory population in the IZ and one "FT-low" population remaining in the SVZ (**Figure 3.2.7D)**. The "FT-high" population corresponds to newly born migratory neurons, as indicated by co-expression of the early neuronal marker NEUROD2. Finally, four days after injection, FT-positive cells can be found in the cortical plate, where they also coexpress NEUROD2 (**Figure 3.2.7E)**.

Our qRT-PCR analysis of FT-labeled cells at various time points after injection was in agreement with our immunostaining analysis (**Figure 3.2.8A-F**). Cells collected at one hour after injection were RGCs that expressed high levels of *Ki67* and *Pax6* and low levels of *Tbr2*. Cells collected after 10 hours were predominantly IPCs that expressed high levels of *Tbr2*. At 24 hours, qRT-PCR confirmed that FACS isolation of the 5-10% highest-expressing cells enriched for immature migratory neurons, which express *Dcx* and *Map2* at higher levels and low levels of *Tbr2*, *Pax6* and *Ki67*. Finally, cells collected after 4 days were neuronal, as the expressed neuronal

transcripts like *Dcx* and *Map2*. Together, these data demonstrate that we can reliably enrich different populations of cortical cells from the excitatory lineage for subsequent downstream analysis.

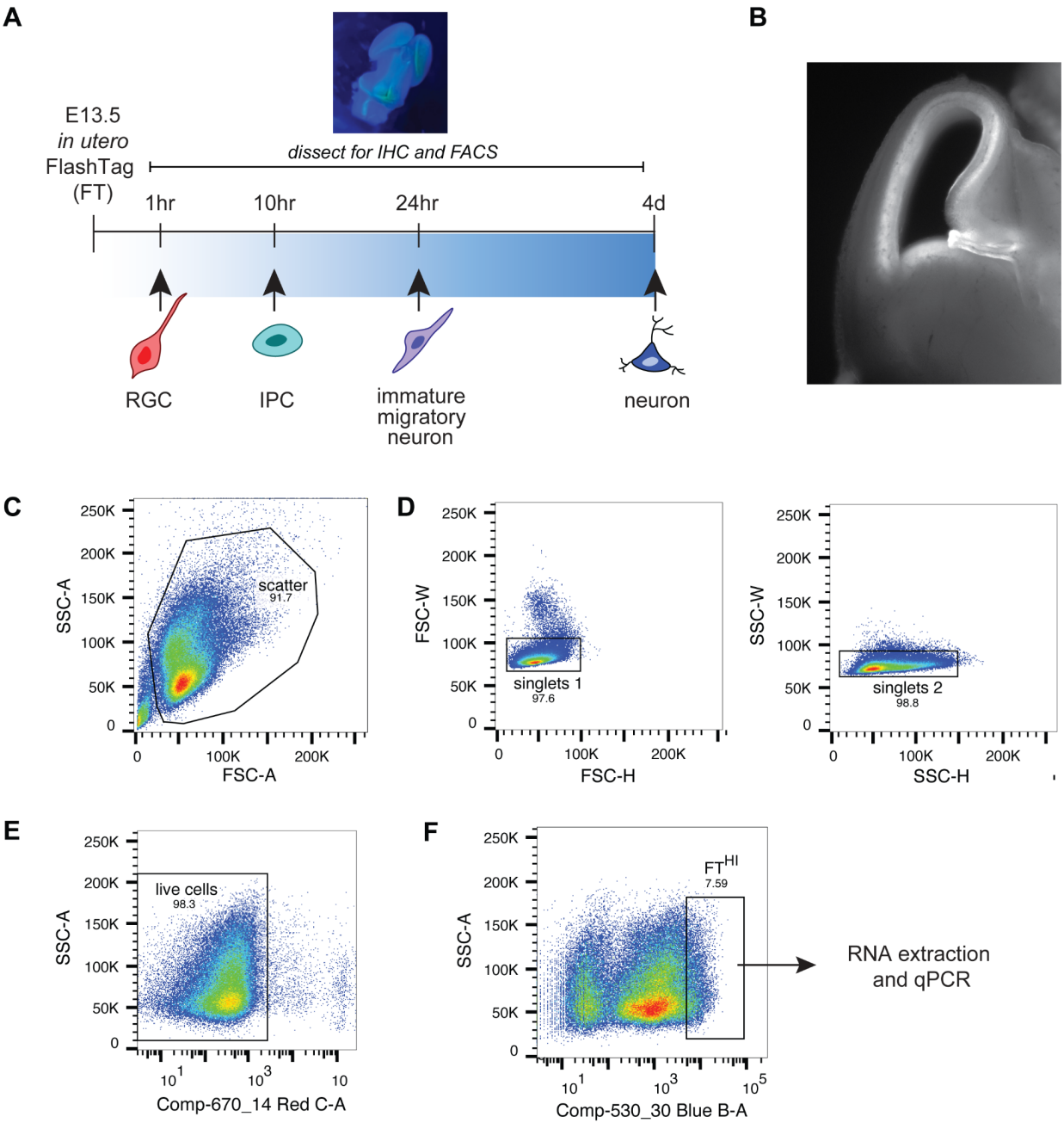

Figure 3.2.6 – FACS isolation of Flashtag (FT)-expressing cortical cells

A) Schematic illustrates the experimental timeline for FT injection and isolation of enriched populations of embryonic cortical cells. FT was delivered via intraventricular injection at E13.5, and FT-labeled cells were collected by FACS at various time points: 1 hour, 10 hours, 24 hours and 4 days to isolate radial glia cells (RGCs), intermediate progenitor cells (IPCs), immature migratory neurons and postmitotic neurons, respectively. Image above timeline depicts a FT-injected brain 1 hour after injection. At this point, FT can be seen in and along the ventricles, fluorescing upon excitation at 488 nm.

B) Image of a FT-injected cortex sectioned on a vibratome 10 hours post-injection. Bright FT-positive cells can be seen in the SVZ.

C-F) Representative FACS plots depicting gates for isolation of FT-labeled cells. The scatter population is first identified using forward and side scatter I. Doublets **(D)** and dead cells, which stain positive for Sytox Red **I**, are excluded from collection. The top 5-10% of FT-positive, live cells are then collected directly into lysis buffer for RNA extraction.

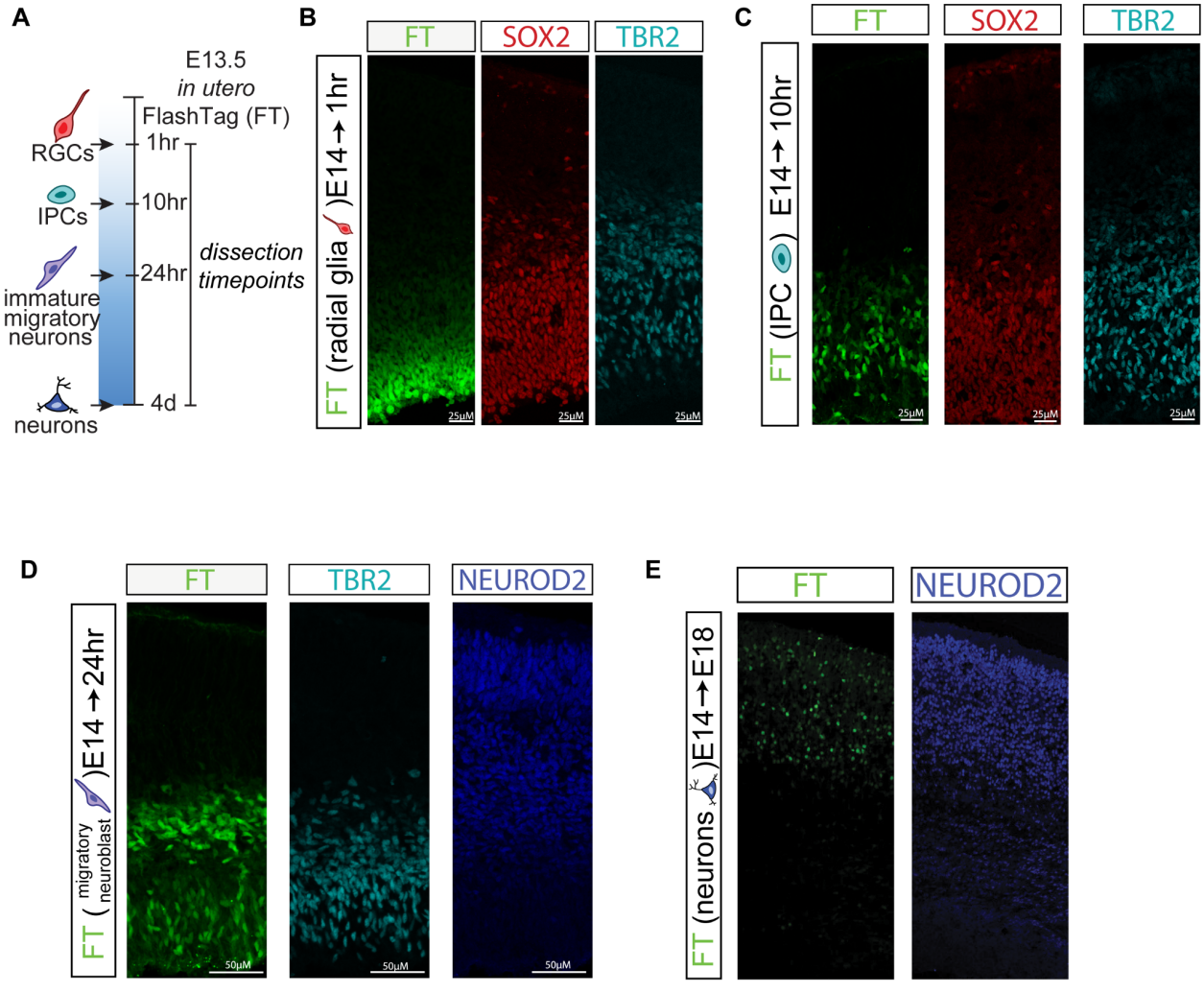

Figure 3.2.7 – Immunofluorescence staining of Flashtag (FT)-expressing cells in embryonic mouse cortices

A) Schematic illustrates the experimental timeline for the isolation of enriched cortical cell populations using FT injection (RGCs, radial glia **cells**; IPCs, intermediate progenitor cells).

B) Representative immunofluorescence images of cortical cells labeled with FT 1 hour after intraventricular injection. FT-labeled cells (green) are enriched in the ventricular zone. Sections were immunostained for SOX2 (red) and TBR2 (teal) to label RGCs and IPCs, respectively. Scale bar, 25μm.

C) Representative images of cortical cells labeled with FT 10 hours after intraventricular injection. FT-labeled cells (green) are enriched in the subventricular zone and co-express TBR2 (SOX2 (red) and TBR2 (teal) label RGCs and IPCs, respectively). Scale bar, 25μm.

D) Representative images of cortical cells labeled with FT 24 hours after intraventricular injection. The brightest FT-labeled cells (green) are enriched in the intermediate zone. Sections were immunostained for TBR2 (teal) and NEUROD2 (blue) to label IPCs and immature neurons, respectively. Scale bar, 50μm.

E) Representative images of cortical cells labeled with FT 4 days after intraventricular injection. Flashtag-labeled cells (green) are enriched in the cortical plate. Sections were immunostained for TBR2 (teal) and NEUROD2 (blue) to label IPCs and neurons, respectively.

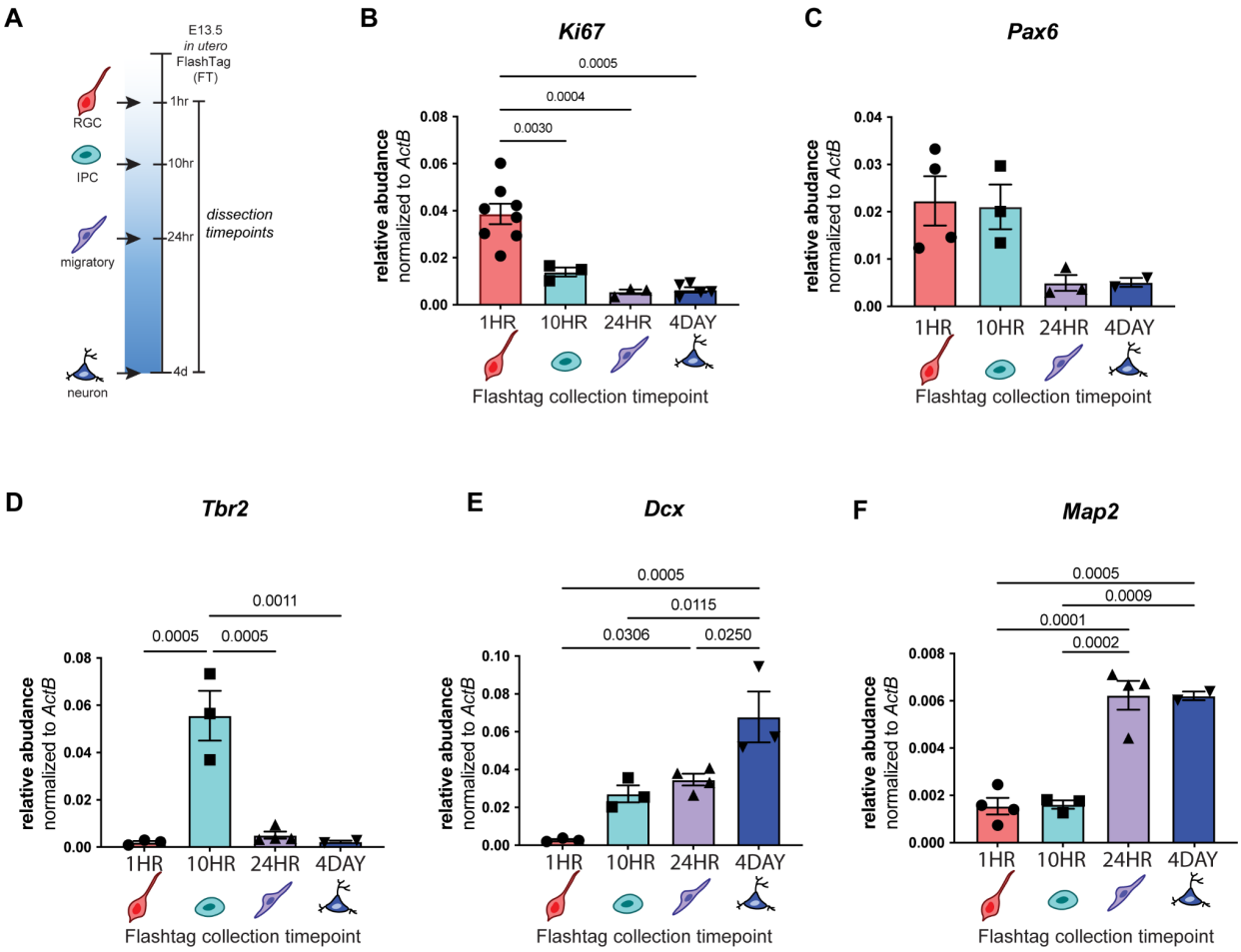

Figure 3.2.8 – Isolating developing cortical populations using Flashtag (FT)

A) Schematic illustrates the experimental timeline for the isolation of enriched populations of cortical cells using FT (RGC, radial glia cells; IPC, intermediate progenitor cells).

B-F) qRT-PCR for cell type-specific transcripts was performed to further validate the identity of FT-labeled populations. RGCs collected at one hour express high levels of the proliferative marker *Ki67* and the RGC marker *Pax6.* IPCs collected at 10 hours continue to express *Pax6* transcripts but are also strongly enriched for high levels of the IPC marker *Tbr2*. Immature migratory neurons collected at 24 hours express high levels of the neuronal markers *Dcx* and *Map2*. Finally, cortical plate neurons, collected 4 days after injection, express high levels of *Dcx* and *Map2* (data presented as mean ± SEM, one way ANOVA and post-hoc Tukey; each data point reflects one-two pooled embryos).

To define the cell type-specific expression of *Orai1-3* in developing cortical populations, we performed qRT-PCR on FT-isolated cells. We found that *Orai1* expression peaked in IPCs (**Figure 3.2.9A)**, while *Orai2* expression was highest in postmitotic neurons (**Figure 3.2.9B)**. *Orai3* was equally expressed in RGCs, IPCs and postmitotic neurons and had the lowest expression

in migratory neuroblasts (**Figure 3.2.9C**). These data are consistent with previously published single cell and bulk datasets and demonstrate dynamic expression of *Orai* transcripts during embryonic cortical development.

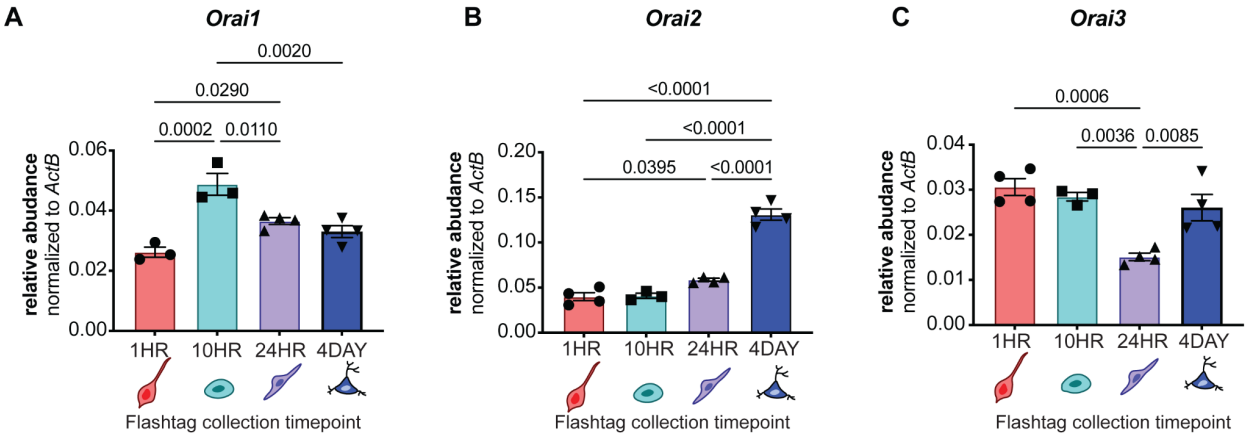

Figure 3.2.9 – *Orai1-3* are dynamically regulated during embryonic cortical development
A-C) qRT-PCR of *Orai1* **(A)**, *Orai2* **(B)** and *Orai3* **(C)** in four different FT-enriched cortical populations. (mean ± SEM, one way ANOVA and post-hoc Tukey; each data point reflects two pooled embryos)

As our expression analysis demonstrated that *Orai1* and *Orai2* were the most highly expressed CRAC channels in the embryonic cortex, we next sought to demonstrate the functional contribution of *Orai1* and *Orai2* to SOCE in primary mouse cortical cells. We bred $Emx1^{Cre}$ mice with mice carrying conditional *Orai1* and *Orai2* alleles ($Orai1^{fl}$;$Orai2^{fl}$), thus generating $Emx1^{Cre}$;$Orai1^{fl}$;$Orai2^{fl}$ mice (or $Emx1^{Cre}$;$Orai1/2^{fl}$) in which *Orai1* and *Orai2* are inactivated in lineages arising from dorsal forebrain radial glia. We then performed SOCE imaging on cortical NSPCs that were isolated from E16 mouse brains. NSPCs were dissociated, plated and transduced with a GcaMP7f lentivirus, as in our initial imaging experiments. While 1μM TG induced comparable ER store depletion in cortical cells from all genotypes, SOCE was significantly attenuated in NSPCs isolated from $Emx1^{Cre}$;$Orai1/2^{fl}$ heterozygous and homozygous mutant mice

(**Figure 3.2.10 A-B**). These results demonstrate that *Orai1* and *Orai2* are necessary for SOCE in embryonic cortical cells.

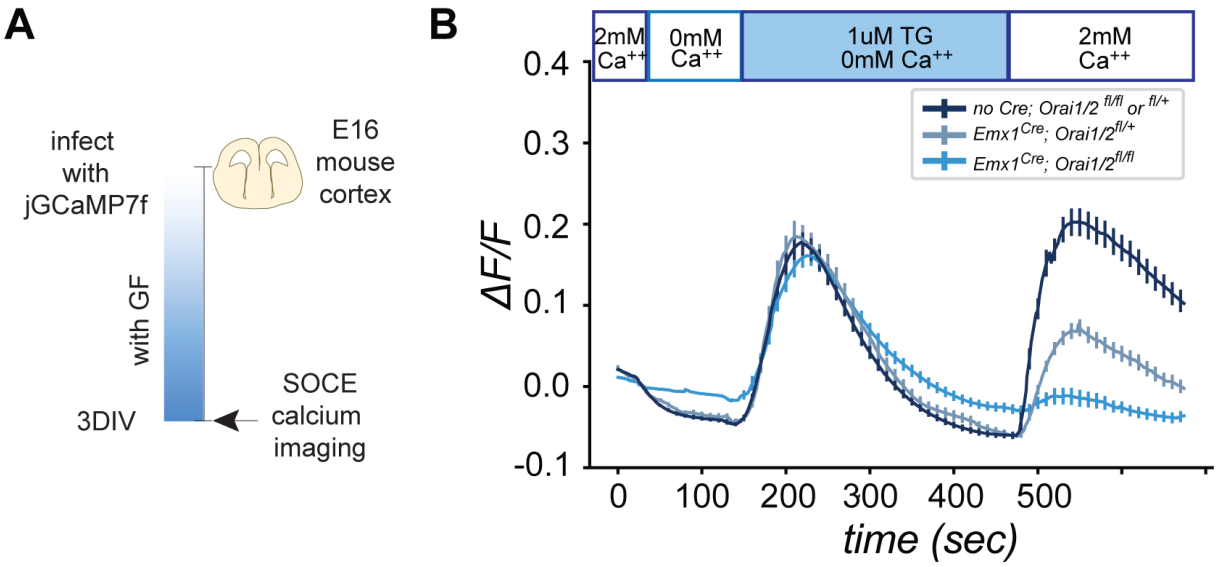

Figure 3.2.10 – SOCE is dose-dependently attenuated in cortical NSPCs from *Emx1^{Cre};Orai1/2^{fl}* heterozygous and homozygous mutant mice
A) Schematic illustrates experimental timeline. E16 NSPCs from control and heterozygous and homozygous conditional knockout mice were transduced with the jGCaMP7f calcium indicator and grown in the presence of growth factors for three days prior to calcium imaging.
B) Mean GcaMP7f imaging traces depict SOCE calcium elevations recorded in control mice (dark blue trace), as well as heterozygous (gray trace) and homozygous (light blue trace)conditional knockout NSPCs. Heterozygous and homozygous mutant cortical cells display significantly reduced SOCE amplitudes in comparison to controls (traces presented as mean ± SEM; control trace: n= 341 cells responding to TG out of 431 total cells; heterozygous mutant trace: n= 268 cells responding to TG out of 407 total cells; homozygous mutant trace: n= 230 cells responding to TG out of 354 total cells).

3.2.3 SOCE is differentially regulated in NSPCs and in postmitotic cells

Thus far, I have shown that the expression of SOCE mediators is dynamically regulated during cortical NSPC lineage progression and that *Orai1/Orai2* are required for SOCE in developing cortical cells. To investigate how SOCE may be differentially regulated in differentiating cells of the developing cortex, we performed post-imaging immunostaining for the

Ki67 antigen, which is present in actively dividing cells, on the same population of mouse NSPCs for which we previously recorded SOCE. We then analyzed SOCE calcium elevations in Ki67-positive and Ki67-negative cells **(Figure 3.2.11A).** My preliminary analyses indicate that SOCE amplitude is significantly higher in actively proliferating, Ki67-expressing NSPCs **(Figure 3.2.11B)**. These data are consistent with calcium imaging performed in differentiating NSPCs from ganglionic eminence-derived neurospheres, which demonstrated that cells negative for the early migratory neuron marker DCX have higher amplitude SOCE rises compared to DCX-positive neuroblasts (Somasundaram et al., 2014).

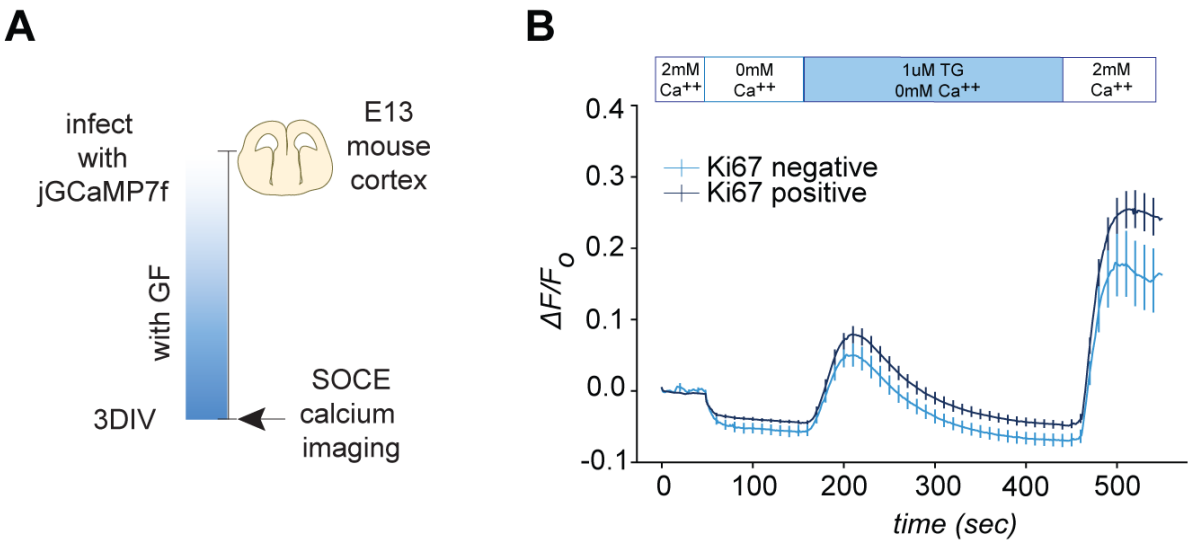

Figure 3.2.11 – Proliferative NSPCs exhibit higher amplitude SOCE than non-proliferative cortical cells
A) Schematic illustrates the experimental paradigm of SOCE calcium imaging experiments performed on E13 embryonic mouse cortical cells maintained in media supplemented with growth factors for 3 DIV.
B) Average GcaMP7f calcium imaging traces for mouse NSPCs that are Ki67-expressing (dark blue) or Ki67-negative (light blue) demonstrate higher SOCE amplitudes in proliferating cortical cells. (traces presented as mean ± SEM; here we include only cells that respond to TG application and display an SOCE rise (i.e., have both a TG and SOCE calcium response) from two coverslips; n= 81 SOCE "responders" out of 85 total Ki67-positive cells, and n= 42 SOCE "responders" out of 60 Ki67-negative cells).

3.2.4 Pharmacological inhibition of SOCE modulates NSPC proliferation in the mouse and human cortex

Our calcium imaging data, which reveal higher amplitude SOCE rises in proliferative cortical cells, support the idea that SOCE may play a role in the regulation of NSPC proliferation in the developing cortex. Indeed, SOCE regulates proliferation in many other cell types, including NSPCs isolated from the ganglionic eminences (Somasundaram et al., 2014), and it has been suggested to regulate aberrant proliferation in disease contexts (Liang et al., 2021). Moreover, early imaging studies have shown that ER store-dependent coordinated calcium transients regulate cortical NSPC proliferation. We thus tested the involvement of SOCE in NSPC proliferation by pharmacologically inhibiting SOCE in cultured NSPCs *in vitro* and subsequently performing flow cytometry-based cell cycle analysis **(Figure 3.2.11A)**. We found that pharmacological inhibition of SOCE using 20μM and 100μM 2-APB in E14 mouse cortical NSPCs led to an increase in the proportion of cells in G0/G1 and accompanying decreases in the proportion of cells in S- and G2/M-phases **(Figure 3.2.11B-E)**. 40μM of AncoA4, a small molecule SOCE inhibitor that specifically destabilizes the ORAI1/STIM1 interaction, also increased the fraction of cells in G0/G1 but did not change the fraction of cells in S- or G2/M-phase, suggesting that ORAI1 and ORAI2 may influence different aspects of the cell cycle or progenitor biology **(Figure 3.2.11B,F)**. Altogether, this data suggests roles for SOCE in the regulation of NSPC proliferation.

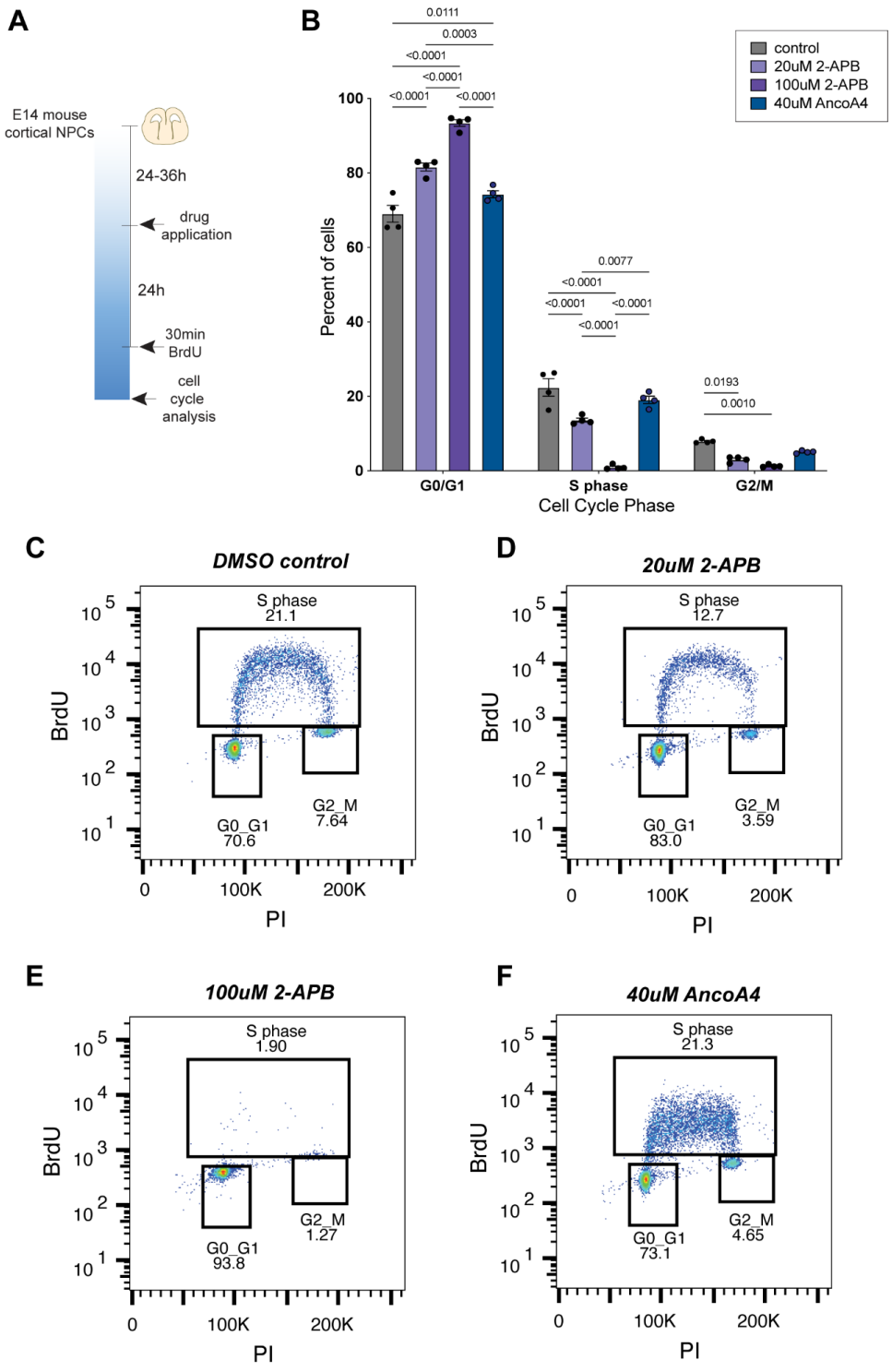

Figure 3.2.12 – Pharmacological inhibition of SOCE reduces proliferation

A) Schematic illustrates the experimental paradigm. E14 cortical NSPCs were dissociated and plated on an adherent substrate in media without GFs. Pharmacological inhibitors of SOCE were applied 24hrs prior to fixation and flow cytometry-based cell cycle analysis. 30 minutes prior to

fixation, cells were pulsed with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) to label S-phase NSPCs.
B) Pharmacological blockade of SOCE in mouse cortical NSPCs reduces the fraction of cells in S-phase, as quantified by FACS analysis for DNA content (mean ± SEM, * $p<0.05$, **** $p<0.0001$, two way ANOVA with post-hoc Tukey; each datapoint represents cortical cells from one pooled litter).
C-F) Representative cell cycle analysis plots from each of the four conditions of NSPCs stained with BrdU and PI to quantify DNA content.

To determine whether SOCE modulation of proliferation is conserved in human NSPCs, we pharmacologically inhibited SOCE in sub-dissected germinal zones from primary GW19-20 human slice cultures and performed FACS-based cell cycle analysis. Similar to mouse cortical cells, we found that the proportion of human cortical cells in G0/G1 increased with SOCE inhibition, while the percentage of cells in S-phase decreased. **(Figure 3.2.11A-C)**. Collectively, these data support that SOCE is necessary for proliferation in both human and mouse NSPCs.

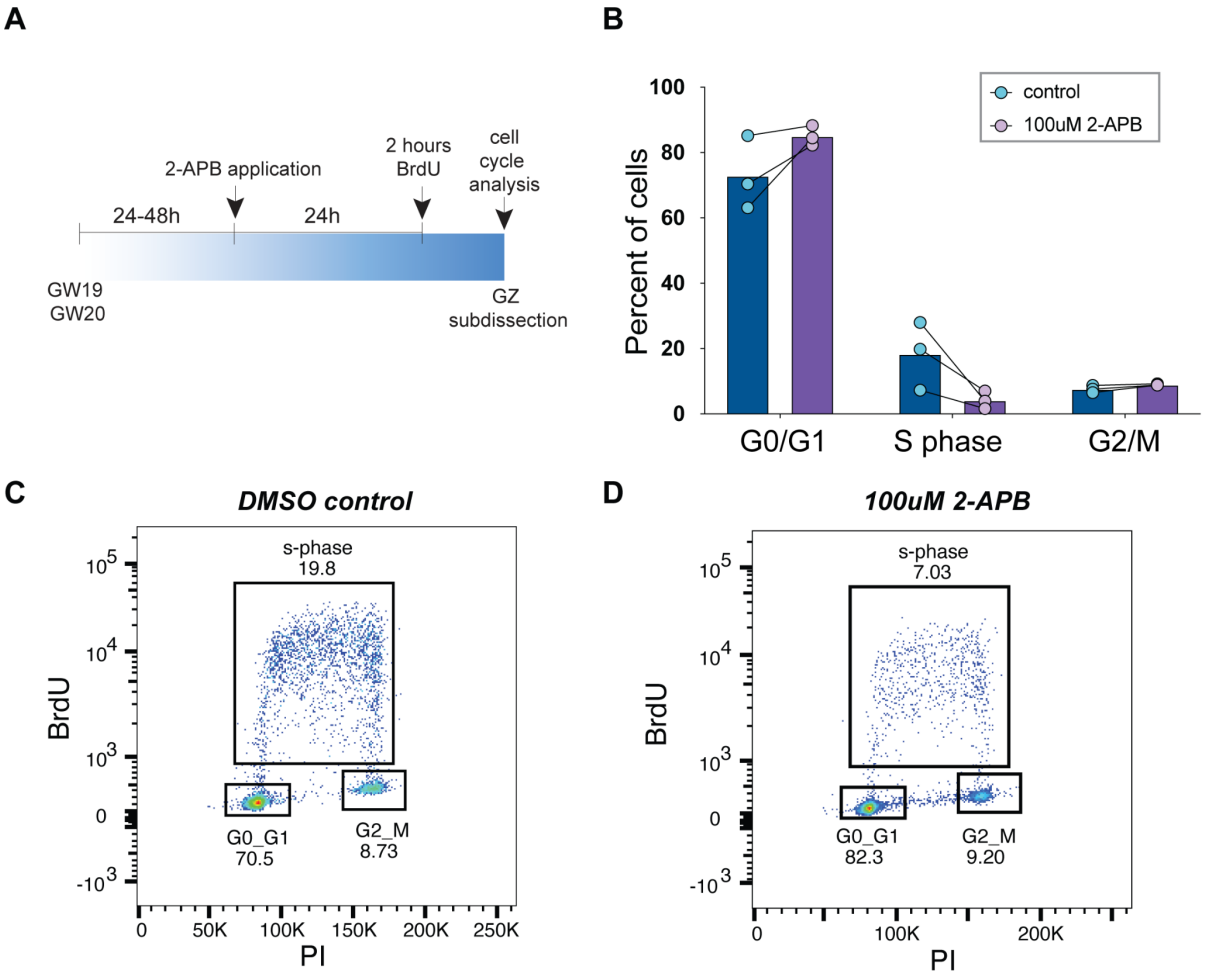

Figure 3.2.13 – Blocking SOCE in human cortical slice cultures reduces NSPC proliferation
A) Representative cell cycle analysis plot of NSPCs treated with a vehicle control and stained with BrdU and PI to quantify DNA content.
B) Representative cell cycle analysis plot of NSPCs treated with 100µM 2-APB and stained with BrdU and PI to quantify DNA content.
C) Blocking SOCE in human cortical NSPCs reduces the fraction of cells in S-phase and increases the proportion of cells in G0/G1, as quantified by FACS analysis (bars represent mean values, paired data points from one human sample treated with vehicle or 2-APB are connected with lines).

3.2.5 ATP signaling initiates SOCE in embryonic cortical cells

In various cell types, ER calcium stores are depleted by cytosolic signaling events that are induced by extrinsic signaling molecules activating plasma membrane receptor tyrosine kinases or

G-protein coupled receptors (Hogan et al., 2010; R. S. Lewis, 2007, 2011; Prakriya & Lewis, 2015). The nature of the extracellular driver of SOCE in the embryonic cerebral cortex, however, remains unknown. Given the possible contribution of SOCE to NSPC proliferation and previous reports of calcium elevations induced by growth factors and other cell cycle regulators (REFs), we performed calcium imaging of NSPCs acutely treated with known positive regulators of proliferation to interrogate potential initiators of SOCE. Epidermal Growth Factor (EGF), for example, is known to promote proliferation and has been shown to initiate SOCE in ganglionic eminence-derived NSPCs (Reynolds et al., 1992; Somasundaram et al., 2014; Tropepe et al., 1999). We investigated whether EGF initiates SOCE in mouse cortical NSPCs by dissociating, plating and transducing E16 cortical cells with lentivirus expressing GcaMP7f. Calcium imaging revealed that acute addition of 100ng/mL EGF to NSPCs imaged in calcium-free media did not elicit cytoplasmic calcium rises, suggesting that EGF does not induce store-dependent calcium rises in E16 NSPCs **(Figure 3.2.14A-C)**.

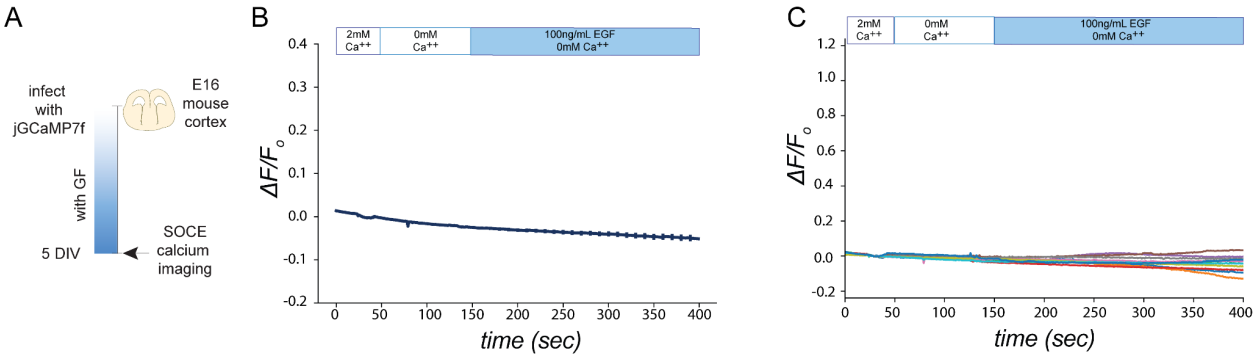

Figure 3.2.14 – EGF does not induce calcium rises in dissociated mouse cortical NSPCs
A) Schematic illustrates the experimental paradigm of SOCE calcium imaging experiments performed on E16+5DIV embryonic mouse cortical cells grown in media supplemented with growth factors.
B) Mean GcaMP7f trace in control cortical cells (*no Cre;Orai1/2*$^{fl/fl\ or\ fl/+}$) acutely treated with 100 ng/mL EGF (n=89 cells, 2 coverslips).
C) Representative traces of calcium rises from individual imaged cells.

Fibroblast growth factor 2 (FGF2/bFGF) is another mitogen that promotes NSPC proliferation and has been shown, at very high concentrations, to induce calcium rises in RGCs (Rash et al., 2016; Tropepe et al., 1999). To interrogate whether bFGF initiates SOCE in embryonic cortical cells, we applied FGF2 in calcium-free media to cortical NSPCs transduced with a lentivirus expressing GcaMP7f. 100ng/mL FGF2, a concentration well above what is typically used to maintain proliferative NSPCs *in vitro*, also failed to induce cytoplasmic calcium rises in our cultures, as with EGF **(Figure 3.2.15A-C)**. Intriguingly, a previous study demonstrated that very high concentrations of FGF2 (1µg/mL) were able to elicit calcium rises in RGC somata and processes in slice cultures of embryonic mouse cortex (Rash et al., 2016). It is likely that the discrepancy between our result and these previous findings results from the vast difference in bFGF concentration used in each experiment. Thus, while it is not clear whether bFGF induces cytoplasmic calcium transients at physiological concentrations, it seems unlikely that it is a primary upstream activator of SOCE in cortical cells.

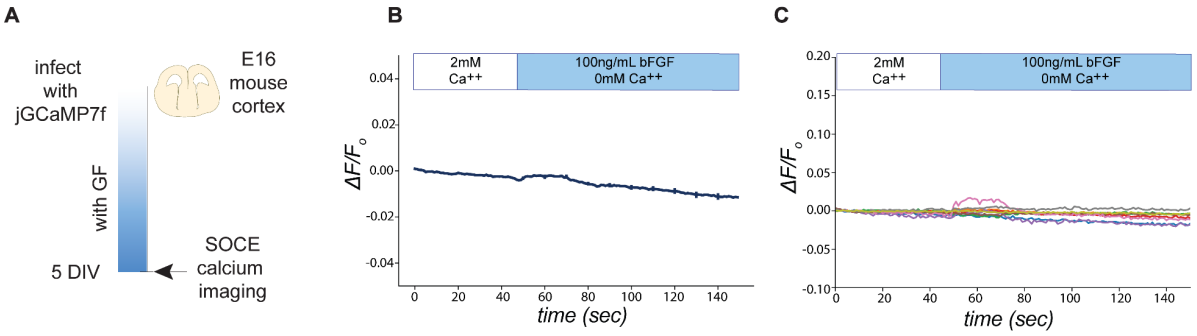

Figure 3.2.15 – FGF2 does not induce calcium rises in dissociated mouse cortical NSPCs
A) Schematic illustrates the experimental paradigm of SOCE calcium imaging experiments performed on E16+5DIV embryonic mouse cortical cells grown in media supplemented with growth factors.
B) Mean GcaMP7f trace in control cortical cells (*no Cre;Orai1/2*$^{fl/fl\ or\ fl/+}$) acutely treated with 100 ng/mL FGF2 (n=61 cells in field of view)

C) Representative traces of calcium rises from individual imaged cells.

In the developing rodent cortex, initiation of coordinated calcium transients is mediated by P2Y1 purinergic receptors (Weissman et al., 2004). *P2Y1R/P2y1r* expression is elevated in ventricular RGCs and downregulated in neurons in the human and rodent cortex (S. Mayer et al., 2019; Weissman et al., 2004), and antagonizing P2Y1Rs inhibits calcium waves and reduces NSPC proliferation (Weissman et al., 2004). As calcium waves are dependent on internal sources of calcium, this makes ATP signaling an attractive candidate to be an upstream regulator of SOCE in the developing cortex. To interrogate this possibility, we dissociated, plated and transduced control (*no Cre;Orai1/2*$^{fl/fl}$ or *Orai1/2*$^{fl/+}$) and mutant (*Emx1*^{Cre}*;Orai1/2*$^{fl/fl}$*)* E16 mouse NSPCs with GcaMP7f-expressing lentivirus (**Figure 3.2.16A,D**). We found that application of the P2Y1 purinergic receptor agonist, MesADP, in calcium-free media induced a robust cytoplasmic calcium rise in both control and mutant NSPCs, likely reflecting a release of calcium from the ER. When 2mM calcium was reintroduced into the media, we saw two primary types of cytoplasmic calcium rises in control NSPCs: the majority of rises were slow and reflected gradual, low amplitude, increases in cytoplasmic calcium levels, whereas a small fraction (~13%) of calcium rises were oscillatory. Importantly, when 2mM calcium-containing media was perfused onto homozygous mutant *Emx1*^{Cre}*;Orai1/2*$^{fl/fl}$ NSPCs, few to no calcium transients were observed **Figure 3.2.16B-C, Figure 3.2.16E-F)**. This finding strongly supports that purinergic signaling induces SOCE in embryonic cortical NSPCs.

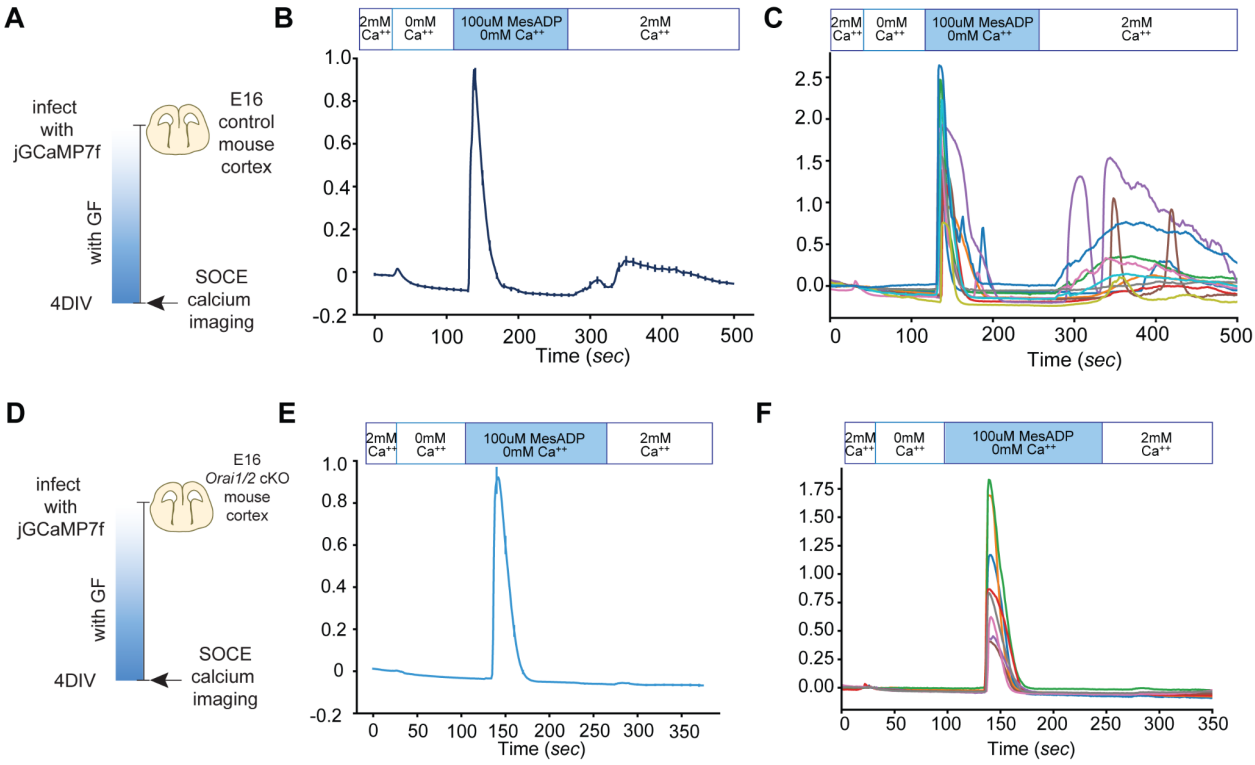

Figure 3.2.16 – Purinergic signaling initiates SOCE in embryonic NSPCs

A) Experimental paradigm for SOCE imaging experiments performed on E16 control (no *Cre*;*Orai1/2*$^{fl/fl}$ or *Orai1/2*$^{fl/+}$) NSPCs.

B) Mean GcaMP7f trace representing calcium elevations in control (no *Cre*;*Orai1/2*$^{fl/fl}$ or *Orai1/2*$^{fl/+}$) cortical cells grown in media supplemented with growth factors. (mean ± SEM; n=113 cells of 147 total cells displaying calcium rises in response to both MesADP and 2mM calcium (SOCE)).

C) Two primary types of SOCE calcium rises can be observed: gradually increasing cytosolic calcium or, less frequently, oscillatory transients. Each trace represents calcium rises from individual representative cells.

D) Experimental paradigm for SOCE calcium imaging experiments performed on E16 E*mx1*^{Cre};*Orai1/Orai2*$^{fl/fl}$ homozygous mutant NSPCs.

E) Average GcaMP7f traces from *Emx1*^{Cre}*;Orai1/2*$^{fl/fl}$ NSPCs. MesADP-induced SOCE is absent in homozygous mutant cells lacking *Orai1* and *Orai2*. (mean ± SEM; n=47 cells of 49 total mutant cells that respond to application of MesADP from one representative coverslip)

F) Imaging traces from individual representative cells reveal that MesADP does not induce SOCE calcium rises in E*mx1*^{Cre};*Orai1/Orai2*$^{fl/fl}$ mutant NSPCs.

3.2.6 Stim2 is the primary calcium sensor in embryonic cortical cells

The STIM family of ER calcium sensors (which consists of STIM1, STIM2 and their splice isoforms) was first discovered to play a role in initiating SOCE through a series of RNAi screens (Liou et al., 2005; Roos et al., 2005). In T cells and mast cells, where the CRAC current was first studied, STIM1 was reproducibly shown to function as the primary ER calcium sensor (Baba et al., 2008; Luik & Lewis, 2007; Oh-Hora et al., 2008; S. L. Zhang et al., 2005). In the developing brain, however, the expression of the STIMs is not known at a cell type-specific level, nor is it clear which STIM serves as the primary ER resident calcium sensor. STIM1 and STIM2 proteins have multiple conserved features. In particular, they each contain: EF-hand domains that extend into the ER lumen and closely interact with a sterile α motif (SAM) domain to enable calcium binding, a transmembrane domain, and a CRAC-activating domain (CAD) domain that allows for interaction with ORAI calcium channels at the plasma membrane (Gudlur et al., 2018; Kawasaki et al., 2009; C. Y. Park et al., 2009; Yuan et al., 2009). Despite their high degree of structural homology, STIM1 and STIM2 also have key differences that render them functionally distinct (Y.-F. Chen et al., 2019; Soboloff et al., 2012; Williams et al., 2001). In comparison to STIM1, for example, STIM2 is able to activate SOCE at lower levels of ER store depletion, likely due to differences in calcium binding affinities and the stability of the EF-SAM domains (Brandman et al., 2007; Stathopulos et al., 2008; L. Zheng et al., 2008). Furthermore, a single amino acid difference in the STIM CAD domains alters their activation profiles, such that STIM2 is a more modest activator of ORAI1 compared to STIM1 (X. Wang et al., 2014). In light of these key distinguishing features, interrogating the expression of the STIM calcium sensors in developing cortical cells is essential to better understanding the mechanistic underpinnings that shape SOCE during differentiation.

To determine the expression patterns of the STIM ER calcium sensors, we first mined existing single cell sequencing datasets from the developing mouse cortex between E12 and E15, which revealed that *Stim2* is more robustly expressed than *Stim1* (**Figure 3.2.17A-C**). To complement this analysis, we also searched the human Brainspan dataset, which revealed high expression of *STIM2* in comparison to *STIM1* prior to birth in human prefrontal cortical samples (**Figure 3.2.18A-C)**. These data suggest that *Stim2/STIM2* is likely the primary ER calcium sensor in the developing cortex.

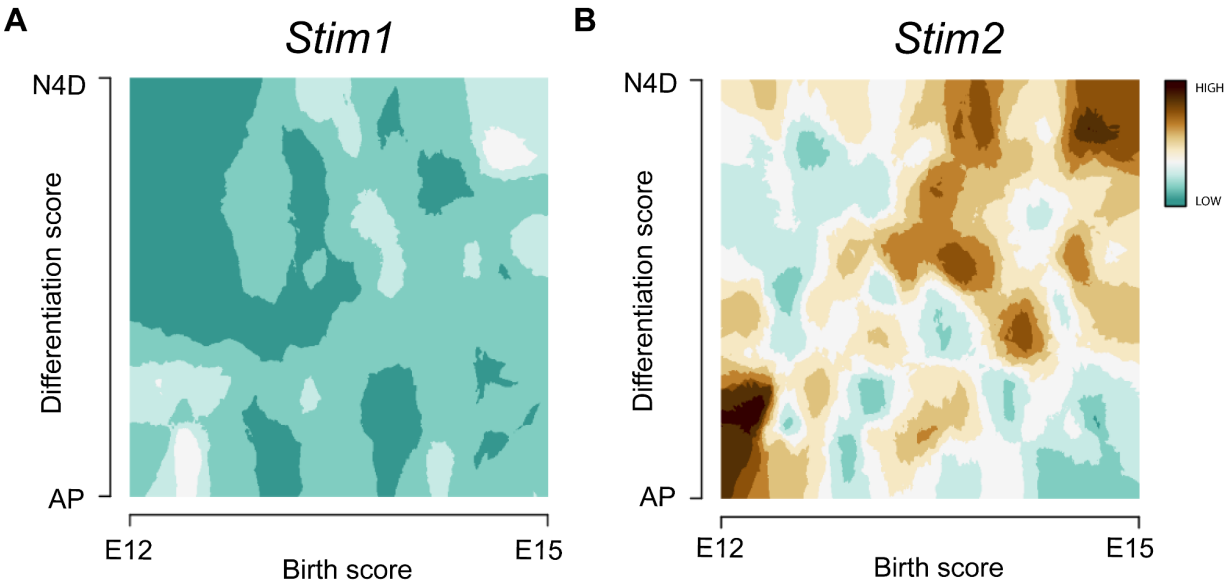

Figure 3.2.17 – *Stim2* is the dominant ER calcium sensor of the mouse embryonic cortex

A) *Stim1 and Stim2* expression extracted from a single cell gene expression dataset obtained from E12-E15 mouse embryonic cortices (E12-E15) by the Jabaudon group (Telley et al., 2019). *Stim2* is highly expressed in the developing cortex. Each cell has a defined birth score (x-axis, E12 to E15 corresponding to the birthdate of isochronic cohorts of cells) and a differentiation score (y-axis, corresponding to differentiation/maturation stage of a given cell ranging from apical progenitor (AP) to postmitotic cortical plate neuron (N4D)) (Telley et al., 2019).

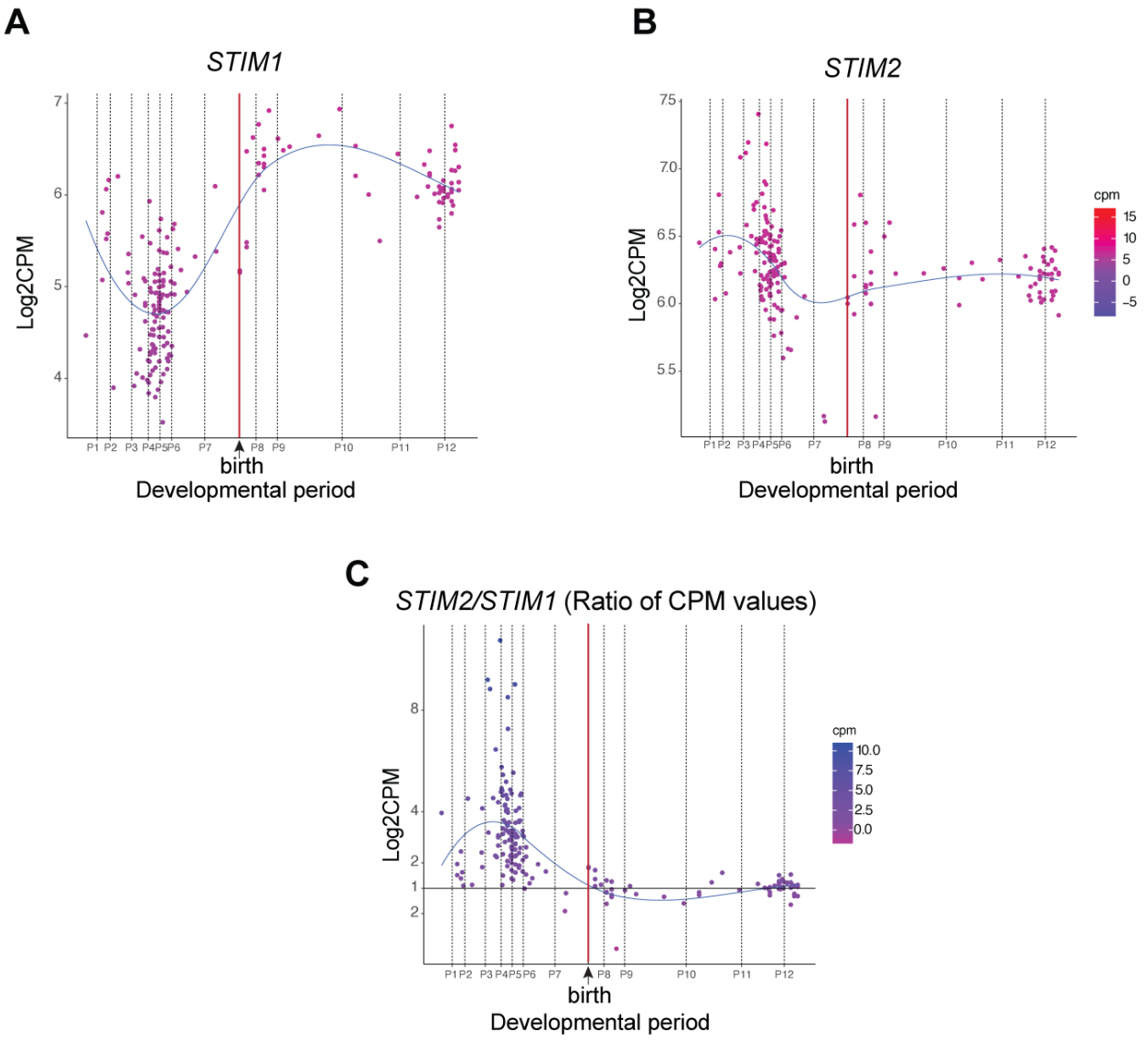

Figure 3.2.18 – STIM2 is the dominant ER calcium sensor of the developing human cortex
A-C) Expression levels in counts per million (CPM) of *STIM1* (**A**), *STIM2* (**B**), and the ratio of *STIM2* to *STIM1* (*STIM2/STIM1*) (**C**) from bulk RNA sequencing of 176 human prefrontal cortical samples ranging from PCW 6 to 20 years of age (Brainspan dataset; stages on x-axis defined as: 3: 10PCW to 13PCW; 4: 13PCW to 16PCW; 5: 16PCW to 19PCW; 6: 19PCW to 24PCW; 7: 24PCW to 38PCW; 8: Birth to 6 months; 9: 6 months to 19 months (Gordon et al., 2021)). Plot in (**C**) was generated by Stephan Sanders.

3.2.2 Stim2α and Stim2β isoforms are dynamically expressed in cortical NSPCs

While STIM1 has been reproducibly characterized as an SOCE activator (Kawasaki et al., 2009; C. Y. Park et al., 2009; Prakriya & Lewis, 2015; Yuan et al., 2009), the literature is full of conflicting reports on the function of STIM2 (Soboloff et al., 2006). Not surprisingly, these have made it difficult to define the contribution of STIM2 to SOCE. It has recently been shown that *Stim2* is differentially spliced into *Stim2α* (or *Stim2.2*) and *Stim2β* (or *Stim2.1*) (Miederer et al., 2015; Rana et al., 2015), two isoforms with distinct effects on SOCE that differ from one another by only 8 amino acids in the CAD domain (Miederer et al., 2015; Rana et al., 2015). Stim2*α* potently activates SOCE, whereas *Stim2β* functions as an SOCE inhibitor. We reasoned that the existence of two splice isoforms with opposing effects on SOCE might explain the conflicting previous findings related to STIM2 function. Since *Stim2* is the primary ER calcium sensor in the developing cortex, we thus interrogated the cell type-specific expression of the *Stim2* isoforms in isolated cortical populations. To this end, we performed qRT-PCR on our FT-enriched samples (**Figure 3.2.6 – Figure 3.2.8**), and we found that *Stim2α* expression increases in postmitotic neurons (**Figure 3.2.19A**). *Stim2β*, on the other hand, is downregulated in immature migratory neurons (**Figure 3.2.19B**). Taking the ratio of *Stim2β* to *Stim2α* (which collectively comprise total *Stim2* levels) reveals an increasing trend in the relative expression of *Stim2β* in IPCs, which is followed by a significant decrease in the immature migratory neuron and postmitotic neuron populations (**Figure 3.2.19C**). These results point to dynamic regulation of *Stim2* isoform expression during NSPC differentiation in the embryonic cortex.

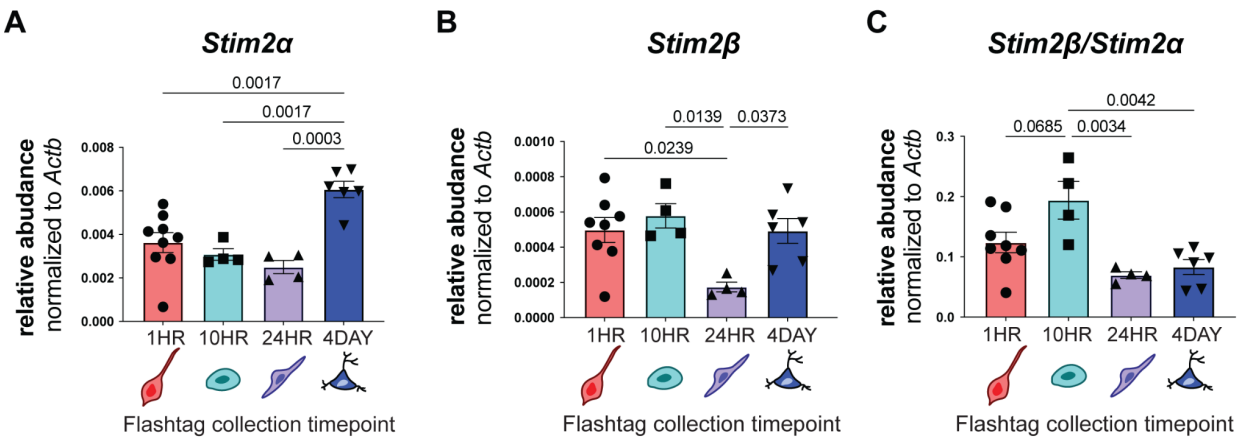

Figure 3.2.19 – *Stim2* variants are dynamically regulated in the embryonic cortex
A-C) qRT-PCR of *Stim2α* **(A)**, *Stim2β* **(B)**, and the *Stim2β/Stim2α* ratio **(C)** in FT-enriched cortical cell populations (mean ± SEM, one way ANOVA and post-hoc Tukey; each data point reflects cortical cells from 1-2 pooled embryos).

3.2.2 Stim2 isoforms bidirectionally regulate cell cycle exit in vivo

Thus far, our data indicate that two isoforms of *Stim2* with opposing effects on SOCE are expressed dynamically during lineage progression in the developing cortex. To interrogate a potential *in vivo* role for these variants, we introduced expression vectors encoding *Stim2α*, *Stim2β,* or *Egfp* into RGCs and their progeny in the E13 mouse cortex using *in utero* electroporation (IUE). We collected electroporated brains at E15 and analyzed region-matched sections for the distribution of electroporated cells across the cerebral wall. To achieve this, we divided the cerebral wall (spanning the VZ to the cortical plate (CP)) into 10 equally-sized "bins" and counted the fraction of electroporated cells in each of these regions (**Figure 3.2.20A-B**). Interestingly, this analysis revealed an increase in the fraction of electroporated cells expressing *Stim2α* in the VZ, suggesting that expression of *Stim2α* may promote proliferation (**Figure 3.2.20C**).

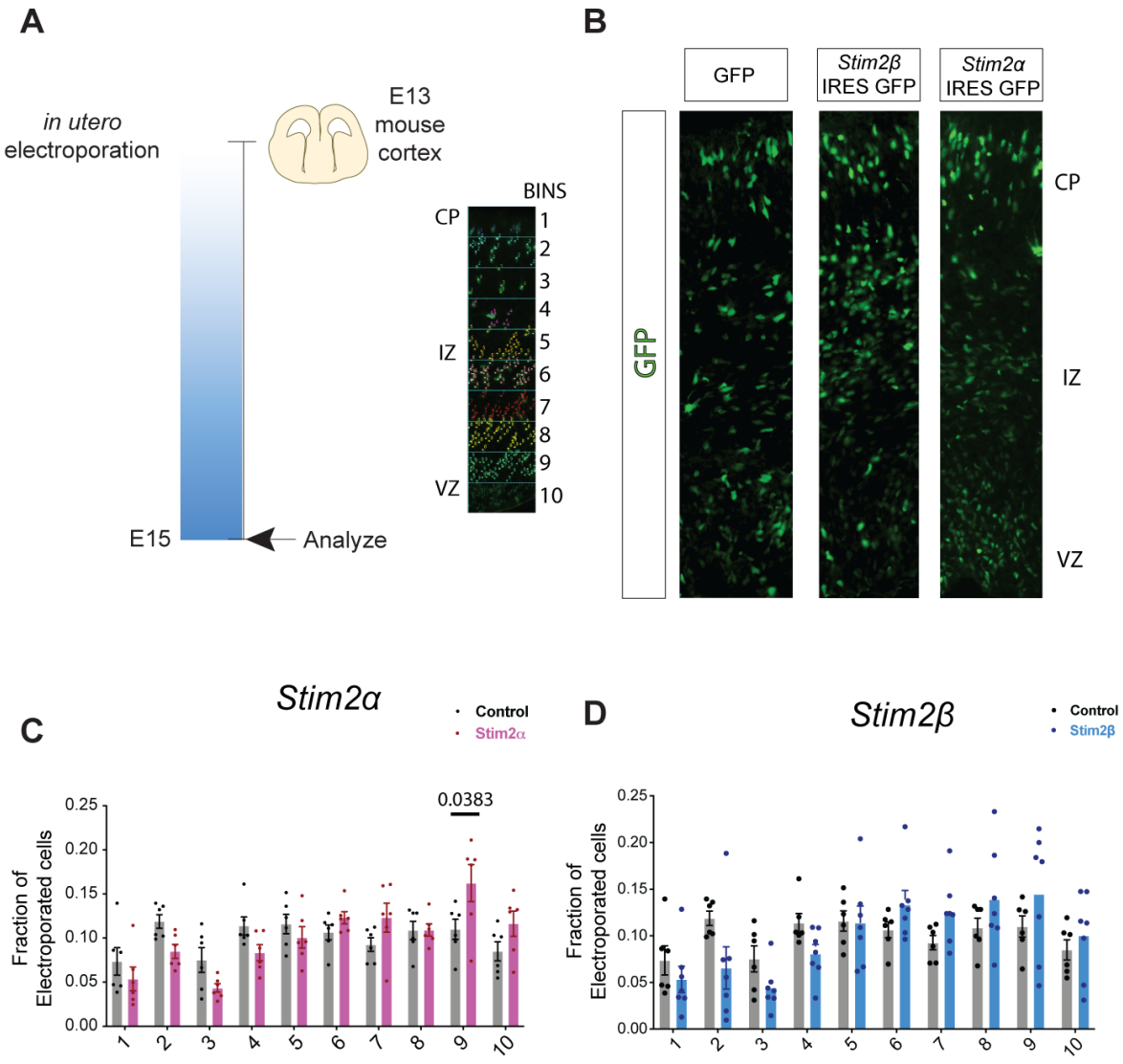

Figure 3.2.20 – *In utero* electroporation of *Stim2α* results in accumulation of cells in the VZ
A) Schematic depicting timeline for *in utero* electroporation (IUE) experiments. Expression vectors encoding *Stim2α*, *Stim2β*, or *Egfp* were introduced into NSPCs in the developing cortex at E13. Brains were collected two days later, at E15, for analysis.
B) Representative images across the cerebral wall of the mouse cortex electroporated with control, *Stim2α*, or *Stim2β*,expression constructs. Electroporated cells in all three conditions are visualized using EGFP.
C) Quantifications of the distribution of electroporated cells across the cerebral wall. A greater fraction of *Stim2α*-electroporated cells remains in the VZ/SVZ two days after cortical IUE. No significant differences in the distribution of cells expressing *Stim2β* were observed (data represent cells from region-matched serial sections; n= 6-7 brains per condition; Bin1=limits of the CP, Bin 10=limits of the VZ; mean ± SEM; * $p<0.05$, two-way ANOVA, post-hoc Bonferroni).

To interrogate potential roles for the *Stim2* splice in the regulation of proliferation, we also quantified the fraction of electroporated cells that exited the cell cycle over a 24 hour period. For this analysis, termed the "quit fraction", expression vectors encoding *Stim2α*, *Stim2β,* or *Egfp* were introduced into the mouse cortex via IUE at E13. At E14, 24 hours prior to sacrifice, we injected pregnant dams with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU), to label all cells in S-phase at the time of injection. We then collected electroporated brains at E15 and performed immunofluorescence staining for antibodies directed against GFP, BrdU and the cell cycle marker Ki67 (**Figure 3.2.21A-B**). Cells that were actively dividing at the time of injection but have since exited the cell cycle will express BrdU but will no longer stain positive for Ki67. We analyzed electroporated cells in region-matched sections across all three conditions and quantified their quit fraction, the ratio of electroporated cells that exited the cell cycle (EGFP+/BRDU+ cells that are negative for Ki67 antigen) over the total number of electroporated cells (EGFP+/BrdU+ cells). 24 hours after the BrdU injection, the fraction of cells that stain positive for BrdU is fairly consistent across conditions (**Figure 3.2.21C**). However, expression of *Stim2β*, the SOCE inhibitor, resulted in elevated quit fraction, whereas expression of *Stim2α*, which activates SOCE, resulted in reduced quit fraction (**Figure 3.2.21D**). These results suggest that modulating SOCE by titrating the expression of these two *Stim2* isoforms was sufficient to bidirectionally control cell cycle exit.

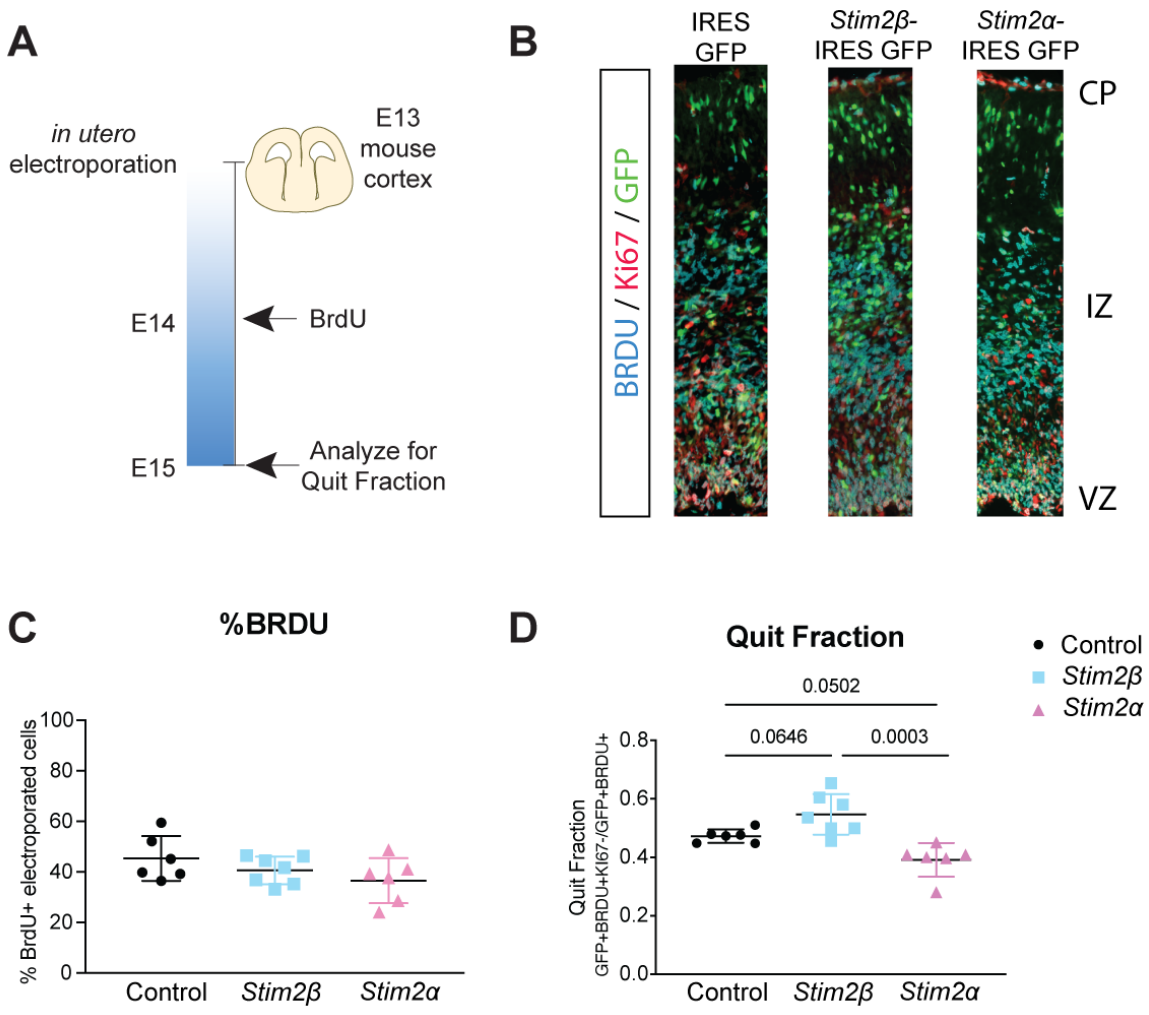

Figure 3.2.21 – *Stim2β* and *Stim2α* bidirectionally regulate cell cycle exit
A) Schematic illustrating experimental timeline. Expression constructs encoding *Stim2α*, *Stim2β,* or *Egfp* were electroporated at E13. BrdU was injected into pregnant dams at E14, and brains were harvested for immunofluorescence analysis of quit fraction 24 hours later.
B) Representative coronal sections through E15 mouse cortices electroporated with control, *Stim2α,* or *Stim2β* expression constructs. Sections were immunostained using antibodies against BrdU (blue), Ki67 (red) and GFP (green).
C) The percentage of *Stim2α*- and *Stim2β*-electroporated cells expressing BrdU is comparable to control.
D) An increased fraction of cells electroporated with *Stim2β* expression vectors exit the cell cycle compared to cells electroporated with an *Egfp* control. In contrast, the fraction of cells exiting the cell cycle is reduced with *Stim2α* electroporation. (data represent cells from region-matched serial sections; n= 6-7 brains per condition; mean ± SEM; one-way ANOVA, post-hoc Tukey).

To begin to interrogate the consequences of *Stim2β* loss of function, we cloned an shRNA construct that specifically knocks down *Stim2β* expression without altering expression of *Stim2α* (**Figure 3.2.22***)*. At E13, we introduced this shRNA construct targeted to *Stim2β* or an shRNA control vector into the cortex via IUE. We then collected electroporated brains at E15 and analyzed region-matched sections from each condition for the distribution of electroporated cells across the cerebral wall, as we did previously with our *Stim2* expression constructs (**Figure 3.2.23A-B***)*. Interestingly, we found that *Stim2β* knockdown results in an accumulation of cells in the VZ/SVZ, accompanied by a reduction in the fraction of cells entering the CP (**Figure 3.2.23A-B***)*. This result partially phenocopied *in utero Stim2α* gain of function (which also resulted in accumulation of cells closer to the ventricle), further supporting a role for these variants in the regulation of cortical proliferation. In particular, when coupled with our overexpression experiments, these findings are consistent with the conclusion that *Stim2β* promotes cell cycle exit.

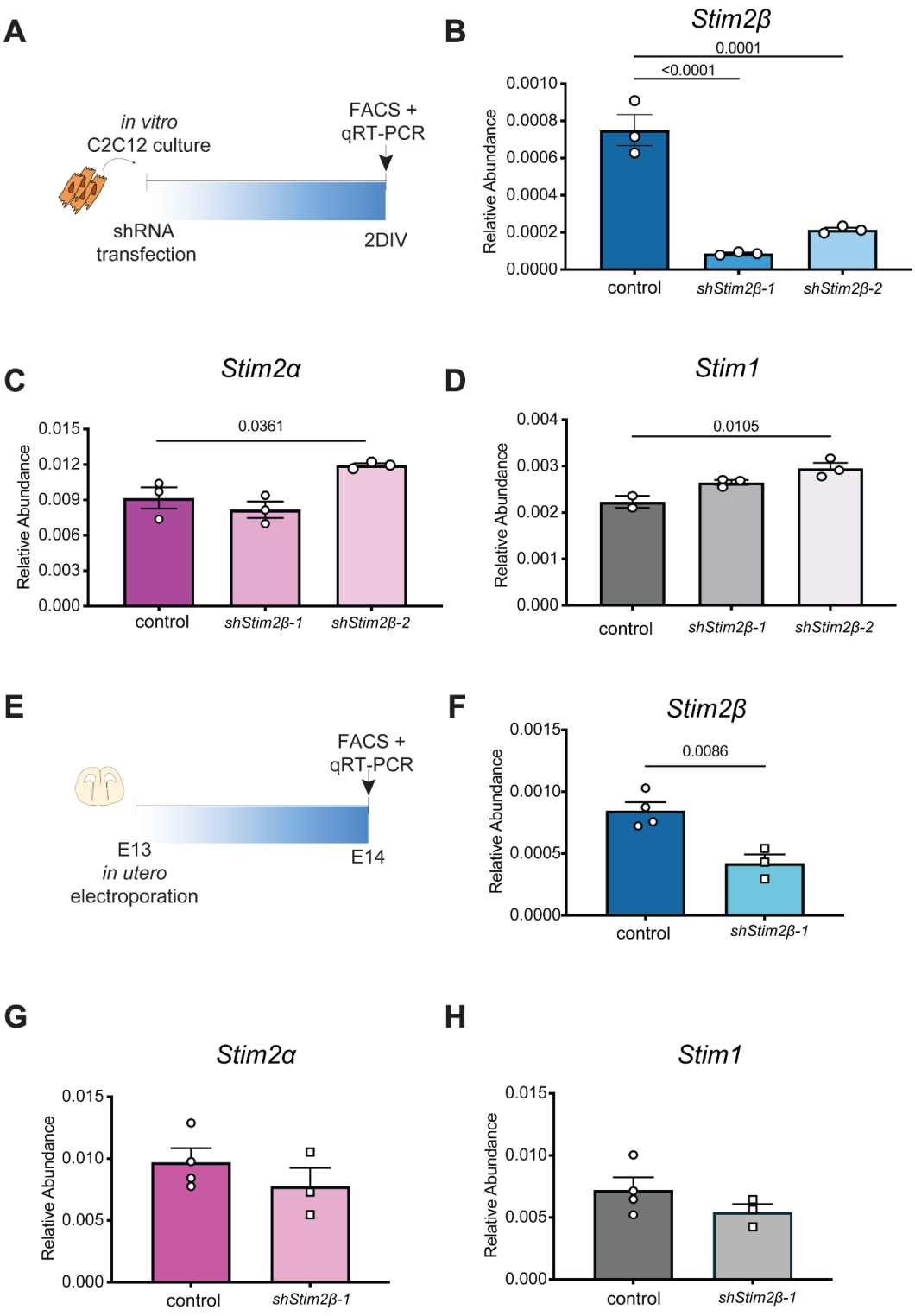

Figure 3.2.22 – *In vitro* and *in vivo* validation of shRNAs directed against *Stim2β*

A) Schematic illustrates experimental timeline for validating shRNA knockdown constructs directed against *Stim2β* in C2C12 cells.

B-D) shRNA construct *shStim2β-1* selectively knocks down *Stim2β* **(B)** without altering *Stim2α* **(C)** or *Stim1* **(D)** mRNA levels in C2C12 cells, assayed using qRT-PCR. (mean ± SEM; one-way ANOVA, post hoc Tukey.)
E) Schematic illustrates experimental timeline for in vivo validation of shRNA knockdown constructs directed towards Stim2β.
F-H) qRT-PCR performed on RNA from FACS-isolated electroporated cells demonstrates that shRNA construct *shStim2β-1* significantly knocks down *Stim2β* **(F)** *in vivo*, without altering *Stim2α* **(G)** or *Stim1* **(H)** mRNA levels. (mean ± SEM; unpaired t-test)

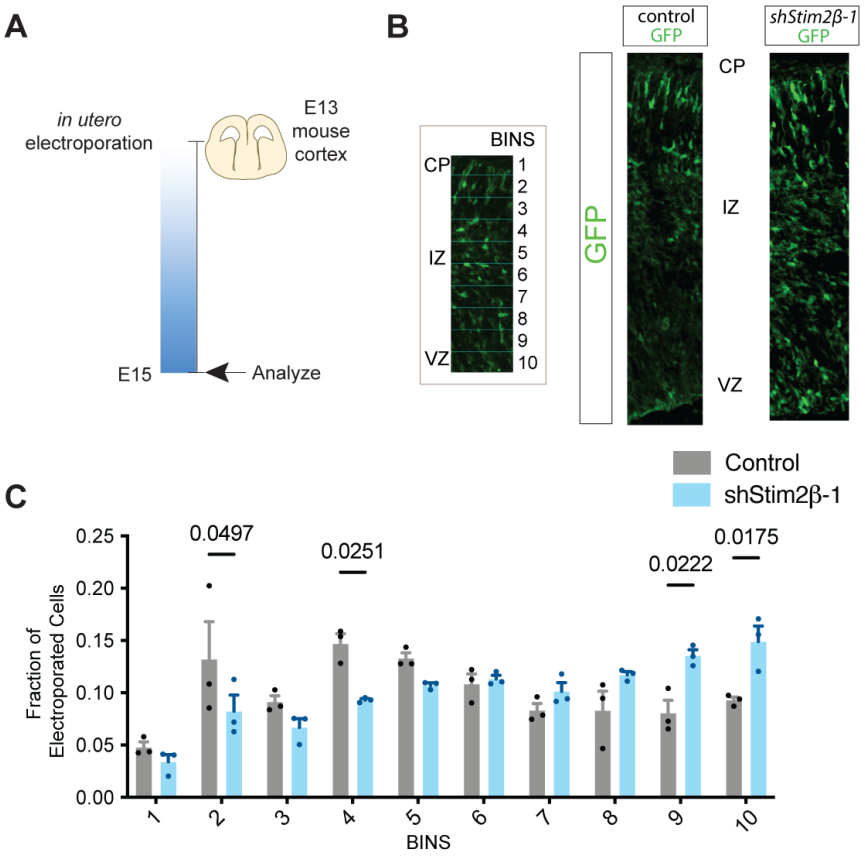

Figure 3.2.23 – *Stim2β* knockdown leads to an accumulation of cells in the VZ/SVZ
A) Schematic illustrates the timeline of IUE experiments testing the *in vivo* effects of *Stim2β* knockdown.
B) Representative images of coronal sections through the mouse cortex electroporated with a control shRNA construct or an shRNA directed to *Stim2β*.
C) Electroporation of *Stim2β* shRNA results in an increased fraction of electroporated cells accumulating in the VZ/SVZ and a reduced fraction of cells reaching the CP. (data represents cells from region-matched serial sections; n=3 brains per condition; Bin1=limits of the CP, Bin 10=limits of the VZ; mean ± SEM.; * $p<0.05$, two-way ANOVA, post-hoc Bonferroni)

3.3 – Discussion and Ongoing/Future Studies

3.3.1 Dynamic regulation of SOCE and its mediators during development

Our data to date demonstrates that 1) SOCE is active in mouse and human cortical NSPCs, 2) that SOCE mediators are dynamically expressed during NSPC lineage progression, 3) that pharmacological blockade of SOCE inhibits proliferation and 4) that *in utero* expression of *Stim2α* and *Stim2β* bidirectionally regulate cell cycle exit of cortical NSPCs. There are, however, some important caveats and fodder for future studies that merit discussion, many centering around the regulation of SOCE and its mediators at different times and in different types of cells during cortical development. Our calcium imaging analyses, for example, were conducted on pooled NSPC populations. A major lingering question is thus whether SOCE is differentially regulated during corticogenesis in RGCs and IPCs, two cell populations whose abundance changes across developmental time. It should be noted that the imaging experiments that first described coordinated calcium transients primarily focused on RGC participation in calcium waves, leaving the contribution of IPCs somewhat unclear (Owens & Kriegstein, 1998; Weissman et al., 2004). Experiments aimed at understanding depolarization-induced calcium influx in developing NSPCs have revealed that cell type-specific effects of extrinsic signals can be masked when studying the NSPC population as a whole (Antonopoulos et al., 1997; LoTurco et al., 1995). Indeed, analyzing the effects of GABA depolarization on VZ and SVZ progenitors separately reveals opposing effects on proliferation in these different NSPC populations (Haydar et al., 2000). Therefore, moving forward we are especially interested in resolving cell-specific SOCE response properties by performing calcium imaging on slice cultures in which IPCs or RGCs have been labeled using Flashtag.

NSPC proliferative behaviors encompass various processes that dynamically vary throughout cell cycle progression, including interkinetic nuclear migration, which involves RGC somata moving up and down within the VZ in phase with the cell cycle, and gap junction coupling. It is thus possible that SOCE is controlled at even further granularity during different phases of the NSPC cell cycle. Since the earliest studies in sea urchin eggs (Mazia, 1937), it has been reproducibly observed that calcium transients may be differentially regulated at the transition between cell cycle phases and thus contribute to cell cycle progression (Berridge, 1995; Owens & Kriegstein, 1998; Poenie et al., 1985, 1986; Volpi & Berlin, 1988; Weissman et al., 2004). Indeed, in cell lines, SOCE is dynamically modulated, such that it is upregulated during S-phase and subsequently attenuated during M (Y.-W. Chen et al., 2016; Smyth et al., 2009). Within the VZ, connexin hemichannels are also essential for calcium wave propagation, suggesting that the dynamics of gap junction coupling during cell cycle progression could contribute to SOCE regulation as well (Bittman et al., 1997; Weissman et al., 2004). In early neurogenesis, NSPCs in the S-, G2- and G1-phases of the cell cycle are connected via gap junctions, whereas in late neurogenesis, cells in S and G1 become progressively uncoupled (Bittman et al., 1997). Moving forward, to fully interrogate SOCE dynamics during NSPC cell cycle phases, we aim to pair calcium imaging of embryonic slice cultures with cell cycle sensors (e.g., FUCCI). Such experiments will further elucidate the temporal and cell state-specific regulation of calcium signals in the developing cortex.

The capacity of ER calcium stores in NSPCs may also change dynamically over developmental time. In line with this, our experiments reveal that the magnitude of the response to thapsigargin differs between E13 and E16 NSPCs. The significance of this finding remains unclear, but it is possible that later RGCs have increased capacity for storing ER calcium in

comparison to early RGCs. Alternatively, this finding may reflect how the composition of the NSPC compartment changes over time, with IPCs increasingly represented in later development. It will be important then to couple cell-specific SOCE calcium imaging with ER-localized calcium indicators to determine how ER contents change over time. It is possible that changes in cell-specific ER calcium content reflect how the three dimensional shape of the ER changes within cortical populations over developmental time. Indeed, dynamic regulation of ER calcium stores has the potential to alter IP3-mediated signaling and the frequency of ER store depletion *in vivo*, thereby altering downstream signal transduction.

Extrinsic regulation of SOCE may also change across embryonic development, which prompts the question of whether SOCE is activated in NSPCs during a specific temporal window in development. For example, ATP signaling, which we identified as a potential upstream activator of SOCE in cortical NSPCs, is temporally regulated as the cortex is built, reaching an apex during the peak of embryonic neurogenesis (Weissman et al., 2004). Thus, even though the magnitude of SOCE does not appear significantly different in our early (E13) and late (E16) primary NSPC cultures *in vitro*, it is plausible that SOCE activation *in vivo* is more dynamic across development. This could be the case both in terms of cell- or cell state-specific expression or activity of SOCE mediators over time, as well as the presence or dynamic activity of upstream environmental signals.

The specific interaction between STIM and ORAI molecules is critical for shaping the characteristics of resultant SOCE rises (Emrich et al., 2021; Yen & Lewis, 2019). X-ray crystallography of the *Drosophila* Orai ortholog first revealed that the CRAC channel is composed of six ORAI subunits (Hou et al., 2012, 2018). STIM interaction with all subunits of the ORAI hexamer at specific stoichiometries is critical for SOCE activation, though there are currently

competing models proposing monomeric *versus* dimeric STIM binding and ORAI crosslinking (Yen & Lewis, 2019). It has recently been shown that different ORAI homologs can associate with one another to form heteromultimers with various calcium influx properties (Yoast, Emrich, Zhang, et al., 2020). Moreover, orchestrated interactions between different STIM and ORAI homologs can result in a diverse array of calcium signaling responses (Emrich et al., 2021). The importance then of characterizing the cell type-specific expression of the *Stim* and *Orai* family members (and their splice variants) in the developing cortex becomes increasingly evident, as interactions between different homologs and variants and their respective stoichiometries that lead to CRAC channel activation can finely tune calcium entry and downstream signaling (Yoast, Emrich, & Trebak, 2020). Along these lines, our calcium imaging experiments and expression analysis on FT–enriched cortical populations demonstrate that *Orai1* and *Orai2* are the primary mediators of SOCE in cortical cells, though their expression changes across lineage progression. Interestingly, we find that proliferative cells (Ki67+) have significantly elevated SOCE amplitudes in comparison to non-proliferative cells. As *Orai2* levels increase with cortical NSPC differentiation **(Figure 3.2.9B)**, it is possible that this difference in SOCE amplitude can be attributed to changes in CRAC channel composition in proliferative and postmitotic cells (Yoast, Emrich, & Trebak, 2020).

3.3.2 How is splicing of the Stim calcium sensors regulated in the developing cerebral cortex?

Our work highlights two splice variants of *Stim2*, *Stim2α* and *Stim2β*, with opposing effects on SOCE, demonstrating that they antagonistically regulate cell cycle exit in the cortex. Alternative splicing of *Stim2* can rapidly change the relative levels of its functionally diverse splice

variants to tune the spatiotemporal properties of SOCE, representing yet another mechanism regulating SOCE-mediated calcium signaling (Miederer et al., 2015; Rana et al., 2015). In order to develop a more complete picture of the dynamic regulation of SOCE in the developing cortex, it will thus be of interest to probe upstream regulators of *Stim2* splicing.

Calcium signaling can be influenced by activity-dependent splicing regulation (Daoud et al., 1999; Panagiotakos & Pasca, 2022). In a preliminary experiment, we tested whether depolarization influenced the splicing of *Stim2* transcripts by dissociating, plating and subsequently depolarizing NSPCs with 67mM KCl for 1 or 6 hours to induce changes in gene expression. As expected, *C-fos* transcript levels significantly increased after KCl-induced depolarization at both 1 and 6 hours. While *Stim1* and *Stim2α* levels did not change using this depolarization paradigm, *Stim2β* was significantly upregulated after 6 hours of KCl-stimulation. These initial results suggest that the splicing of *Stim2* may be regulated by electrical activity.

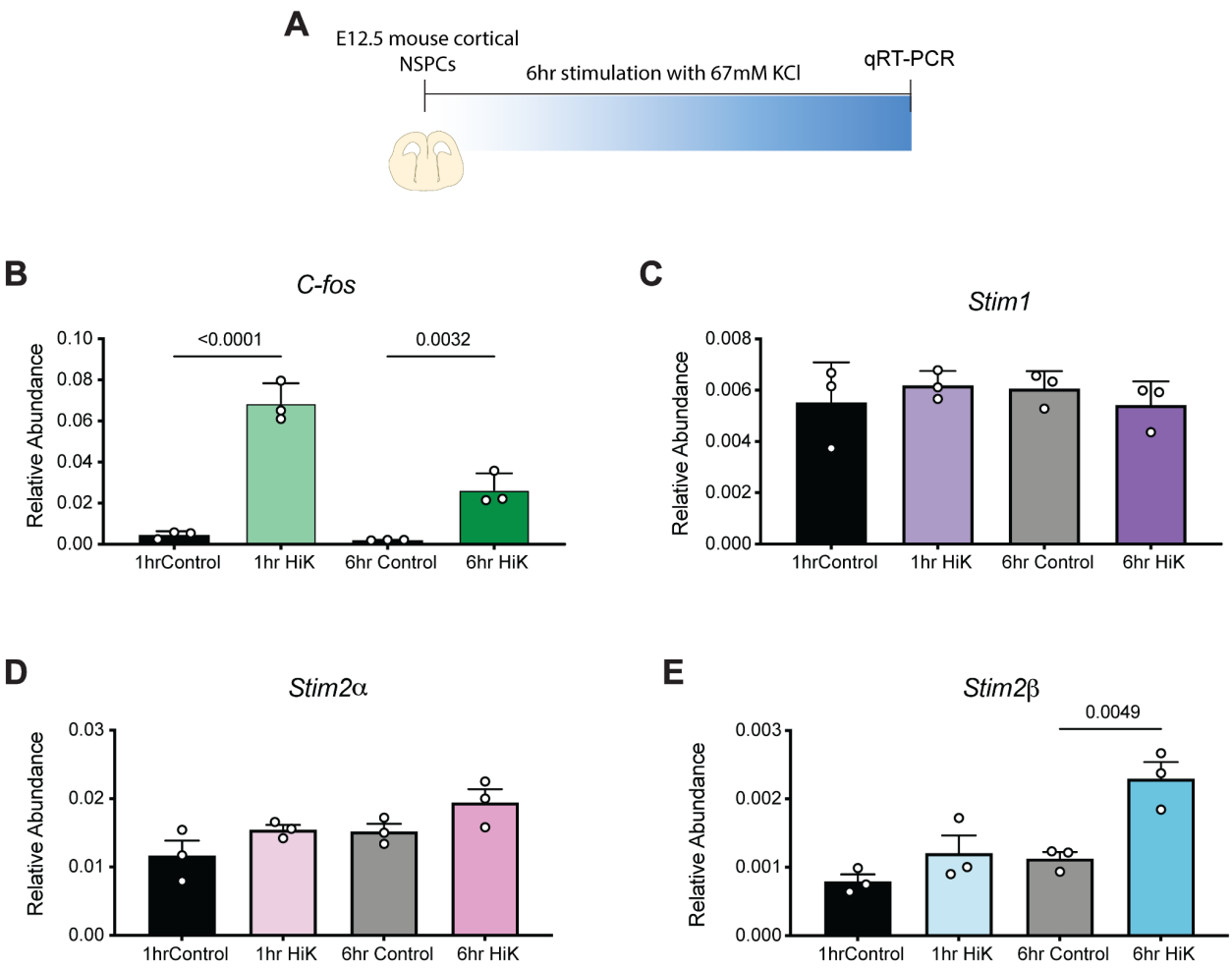

Figure 3.3.1 – *Stim2β* splicing may be activity regulated.

(A) Schematic illustrates the experimental timeline. Briefly, E12.5 NSPCs were dissociated, plated, and depolarized with 67mM KCl (HiK) for 1 or 6 hours to induce activity-dependent changes in gene expression.

(B-E) qRT-PCR quantifying the relative abundance of *C-fos* **(B)**, *Stim1* **(C)**, *Stim2α* **(D)**, and *Stim2β* **I** transcripts in NSPCs demonstrates elevated expression of *Stim2β* upon depolarization. (mean ± SEM.; one way ANOVA, post-hoc Sidak)

Activity is an emerging regulator of alternative splicing in the developing cerebral cortex (An & Grabowski, 2007; Iijima et al., 2011; Q. Li et al., 2007; Xie & Black, 2001) and alterations in neuronal activity in the context of neurodevelopmental disease are linked to abnormal alternative splicing (Eom et al., 2013; Irimia et al., 2014; Quesnel-Vallières et al., 2016; Vuong et al., 2016). Importantly, alternative splicing has been shown to regulate key mediators of various

aspects of cortical development, including neuronal migration and differentiation, cell-fate acquisition, neurite extension and dendritic retraction (Boutz et al., 2007; Kamijo et al., 2018; Krey et al., 2013; Licatalosi et al., 2012; Linares, 2015; Linares et al., 2015; Makeyev et al., 2007; Panagiotakos et al., 2019; Panagiotakos & Pasca, 2022; Paşca et al., 2011; Tang et al., 2011; X. Zhang et al., 2016; S. Zheng et al., 2012). Alternative splicing may therefore represent a global mechanism deployed during development to dynamically control calcium signaling and calcium-regulated cellular behaviors.

3.3.2 SOCE and NSPC proliferation in the embryonic cerebral cortex

Our pharmacological experiments in both mouse and human primary tissue suggest that SOCE regulates NSPC proliferation in the embryonic cortex. These results are in agreement with studies performed in neurosphere cultures of ventrally derived NSPCs, which demonstrate that SOCE is a regulator of embryonic NSPC proliferation (Somasundaram et al., 2014). Studies in other tissues and in diseases of abnormal proliferation have also implicated SOCE in cell cycle regulation (Ay et al., 2013; Y.-F. Chen et al., 2011; Kim et al., 2019; G. Li et al., 2013; Motiani et al., 2013; Umemura et al., 2014; Xie et al., 2016). Interestingly, in our experiments, pharmacological inhibition of SOCE with both 2-APB and AncoA4 reduced mouse NSPC proliferation at E14 but had diverse effects on the regulation of different cell cycle phases, suggesting that SOCE regulation of proliferation may be more complex (e.g., perhaps SOCE influences the duration of distinct cell cycle phases). One potential caveat that must be addressed as an immediate next step requires rigorous characterization of cell death in the context of SOCE inhibition. High concentrations of 2-APB (100 μM) yielded increased cell death in our dissociated mouse cortical cultures *in vitro* (data not shown). In our experiments, we thus used lower

concentrations of 2-APB, which effectively inhibit SOCE (Prakriya & Lewis, 2001; X. Zhang et al., 2020), to minimize any possible cell death-related phenotypes. Our preliminary analysis of *Emx1*^{Cre}*;Orai1*^{fl}*;Orai2*^{fl} mutant cortices have also not revealed any significant alterations in apoptosis (data not shown); nevertheless, a thorough quantification of the abundance of apoptotic cells is required.

Also consistent with the idea of SOCE as a cell cycle regulator is the finding that ATP signaling, which has been shown to influence BrdU uptake in cells of the developing cortex, can promote SOCE in cortical cells *in vitro.* Indeed, ATP-induced calcium waves, mediated by intracellular stores of calcium, are necessary for NSPC proliferation in slice preparations of the rodent embryonic cortex (Weissman et al., 2004). It will be essential to interrogate the effects of ATP on proliferation in the context of *Orai* deletion, in order to mechanistically link ATP to SOCE-mediated proliferation. Importantly, as the list of candidate SOCE regulators that we tested was not comprehensive in scope, it will be important to screen in a more unbiased fashion for other possible extracellular signaling molecules that initiate store-dependent calcium influx at different embryonic time points. Moving forward, we must also confirm, using ER-localized calcium indicators, that potential upstream activators of SOCE do indeed deplete ER calcium stores.

The observed effects of the *Stim2* isoforms on NSPC cell cycle exit further suggest that modulating calcium via SOCE can bidirectionally regulate proliferation. In comparison to *Stim2α*, *Stim2β* contains an additional exon that disrupts the CAD domain, destabilizing its interaction with ORAI channels and thus rendering it an inhibitor of SOCE (Miederer et al., 2015; Rana et al., 2015). Interestingly, *Stim2β* is widely expressed across different tissues and is evolutionarily conserved. The relative expression of *Stim2β* to *Stim2α* is dynamically regulated in differentiating myoblasts and in I and stimulated T-cells, suggesting that their expression may be strictly regulated

to tune SOCE during various cellular processes (Kim et al., 2019; Miederer et al., 2015; Rana et al., 2015). In the developing cortex, we found that the *Stim* isoforms are dynamically expressed in a cell-type specific manner and that they influence cell cycle exit in opposing directions. *Stim2α* levels increase in cortical plate neurons, paralleling overall increases in *Orai2* levels with lineage progression, whereas *Stim2β* is specifically downregulated in the migratory immature neuron population. Interestingly, we observed an increasing trend in the ratio of *Stim2β* to *Stim2α* between the RGC and IPC populations, suggesting that *Stim2β* may be modestly upregulated to participate in IPC cell cycle exit. Alternately, *Stim2β* upregulation may be required to specify one choice of RGC daughter cell over another. It will be of great interest to collect FT-labeled populations at different embryonic time points to determine how the *Stims* are regulated in the same cell populations across time. As the contribution of ATP signaling, the degree of RGC gap junction coupling, and the number of cells participating in calcium waves increases over developmental time, it is possible that the modest effects we see with *Stim2* gain of function *in utero* are enhanced at later time points. To investigate this possibility, we have begun manipulating the *Stim2* isoforms *in utero* at E15, coinciding with the peak of neurogenesis and the birth of upper layer neurons in the mouse.

Indeed, the exact roles that the *Stim2* isoforms play in proliferative NSPCs remain unclear. We plan to conduct live imaging of electroporated cortical slices expressing gain- and loss-of-function *Stim* variant constructs in combination with cell cycle sensors (e.g., FUCCI) to further elucidate their potential functions at discrete phases of the cell cycle. To more fully characterize the effects of *in utero* overexpression of *Stim2α* and *Stim2β* on cell cycle exit, it will also be important to perform immunohistochemical analysis of the cells that are prematurely exiting the cell cycle to determine whether they are differentiating into neurons of different types. In addition,

a more complete analysis of brains electroporated with shRNA directed against *Stim2β* is needed to assess cell cycle exit and other metrics associated with NSPC proliferation. As our shRNAs reduce but do not completely abrogate *Stim2β* expression, alternate loss-of-function strategies (e.g., CRISPR/Cas) may be required to fully reveal the function of this isoform. Finally, as the expression of *Stim2α* increases with neuronal differentiation, it will be of great interest to dissect the effects of the *Stim2* variants on later developmental cellular behaviors, including neuronal differentiation. We can achieve this by restricting expression of *Stim2* variants to postmitotic neurons (using specific plasmid backbones) and interrogating distinct aspects of neuronal differentiation, including neurite elaboration, molecular identity, axonal projections and electrical activity.

3.3.3 What are the consequences of Orai1 and Orai2 inactivation in the developing mouse cortex?

Our calcium imaging and expression analyses suggest that *Orai1* and *Orai2* are the primary regulators of SOCE in the embryonic cortex. In order to investigate whether these SOCE regulators contribute to the regulation of NSPC proliferation, we inactivated *Orai1* and *Orai2* in dorsal forebrain RGCs and their progeny by crossing $Emx1^{Cre}$ mice to $Orai1/2^{fl}$ mice (see methods for details). We collected brains at E17 following a two hour pulse with BrdU to label cells in S-phase. We then counted region matched sections through the lateral cerebral wall for the total number of BrdU-positive cells spanning the region from the VZ to the pial surface. We found no significant change in the absolute number of BrdU positive cells between control mice (no *Cre*;$Orai1/2^{fl/+}$ or $^{f/f}$) and $Emx1^{Cre};Orai1/2^{fl}$ heterozygous and conditional knockout mutants. Notably, it is possible

that the total number of cells is altered in these mice, altering the fraction of BrdU-positive cells, so it will be essential to quantify total cell number and proportion of BrdU cells moving forward.

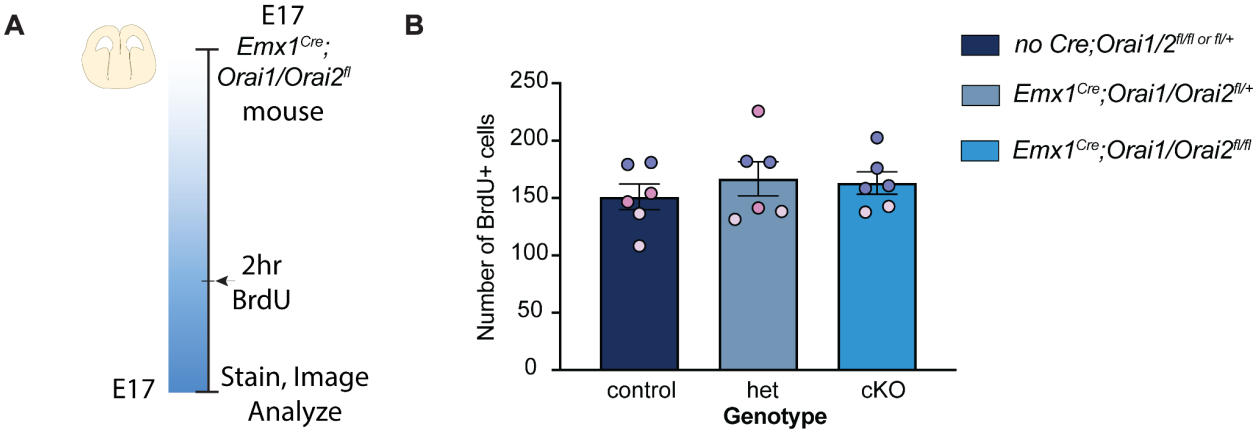

Figure 3.3.2 Cortex-specific conditional deletion of *Orai1/2* does not change the absolute number of BrdU+ cells in the cortex at E17
A) Schematic illustrating experimental timeline. E17 *Emx1^{Cre};Orai1/2^{fl}* brains were harvested for immunofluorescence analysis 2hrs after BrdU injection into pregnant dams. Immunofluorescence staining was performed to label BrdU+ S-phase cells.
B) No differences in the number of BrdU positive cells *Emx1^{Cre};Orai1/2^{fl}* control, heterozygous and cKO animals. (data represent cells from region-matched serial sections; cells counted from a 120 micron window that spanned the VZ to the pial surface; similar colored datapoints reflect animals from the same litter; n= 6 brains per condition; mean ± SEM; one-way ANOVA).

As an orthogonal approach to quantify the fraction of BrdU-positive cells in the cortex at E17, we pulsed pregnant dams with BrdU for 2 hours, dissected cortices, and prepared single cell suspensions for flow cytometry-based cell cycle analysis, recognizing known limitations of this approach for detecting small differences in proliferation. In accordance with our immunofluorescence analysis of BrdU in the E17 cortex, our preliminary cell cycle analysis revealed no significant difference between the percentage of cells in each phase of the cell cycle at E17. Importantly, at this later stage of corticogenesis, flow cytometry-based cell cycle analysis after a two-hour pulse of BrdU captures a very small fraction of cells in S-phase, raising concerns that this approach may be hitting against a lower limit of detection and is not optimal for detecting

subtle changes in NSPC proliferation. Nevertheless, these preliminary results suggest that deleting both *Orai1* and *Orai2* in cortical NSPCs does not alter gross measures of proliferation.

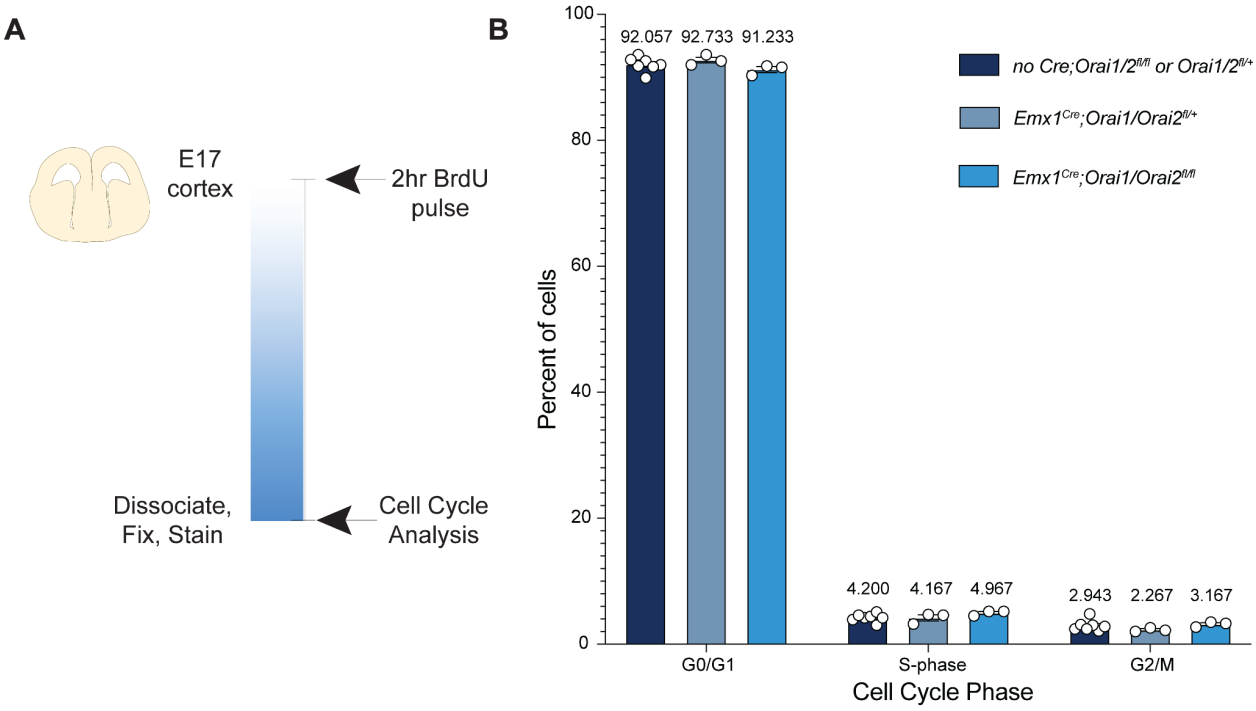

Figure 3.3.2 - Cell cycle analysis of E17 *Emx1^{Cre};Orai1/2^{fl}* cortices
A) Schematic illustrates experimental paradigm. E17 *Emx1^{Cre};Orai1/2^{fl}* timed-pregnant dams were injected with BrdU to label S-phase NSPCs. 2 hours after injection, E17 cortices were dissected, dissociated, fixed and stained for flow cytometry-based cell cycle analysis.
B) Our preliminary results indicate no change in the fraction of cells in G0/G1, S-phase, or G2/M, as quantified by flow cytometry-based cell cycle analysis for DNA content (each datapoint represents cortical cells from one E17 embryo; mean ± SEM).

We also quantified the fraction of BrdU-positive cells in E15 cortices by flow cytometry-based cell cycle analysis. To this end, we pulsed *Emx1^{Cre};Orai1/2^{fl}* timed-pregnant dams with BrdU for 30 minutes, and subsequently dissected cortices into single cell suspensions and stained cells for cell cycle analysis. Our preliminary analysis reveals no significant difference in the percentage of cells at each cell cycle phase. Once again the total proportion of BrdU+ cells (as a percent of total cells in the E15 cortex) is relatively low; therefore, it will be essential to supplement

this analysis with immunofluorescence staining to identify any small changes in S-phase cells as a fraction of all cycling cells in cortices lacking *Orai1* and *Orai2*.

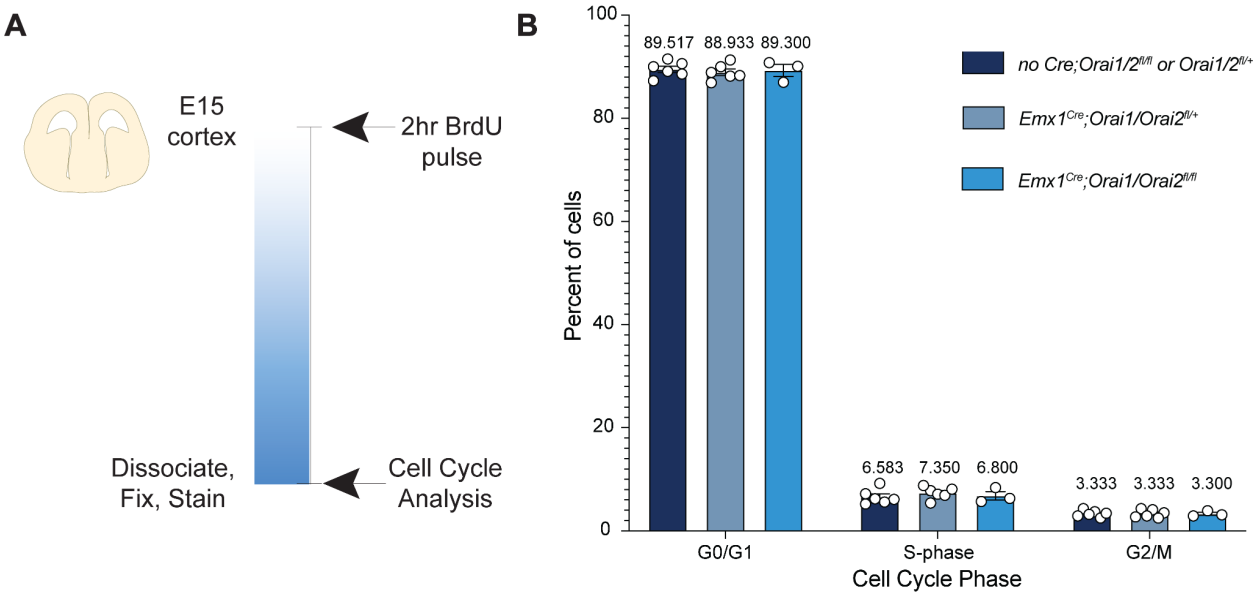

Figure 3.3.3 - Cell cycle analysis of E15 *Emx1^{Cre};Orai1/2^{fl}* cortices
A) Schematic illustrates the experimental paradigm. E15 *Emx1^{Cre};Orai1/2^{fl}* timed-pregnant dams were injected with BrdU to label S-phase NSPCs. 30 minutes after injection, E15 cortices were dissected, dissociated and fixed for flow cytometry-based cell cycle analysis.
B) Our preliminary results indicate no change in the fraction of cells in G0/G1, S, or G2/M phases, as quantified by flow cytometry-based cell cycle analysis for DNA content (each datapoint represents cortical cells from one E15 embryo; mean ± SEM).

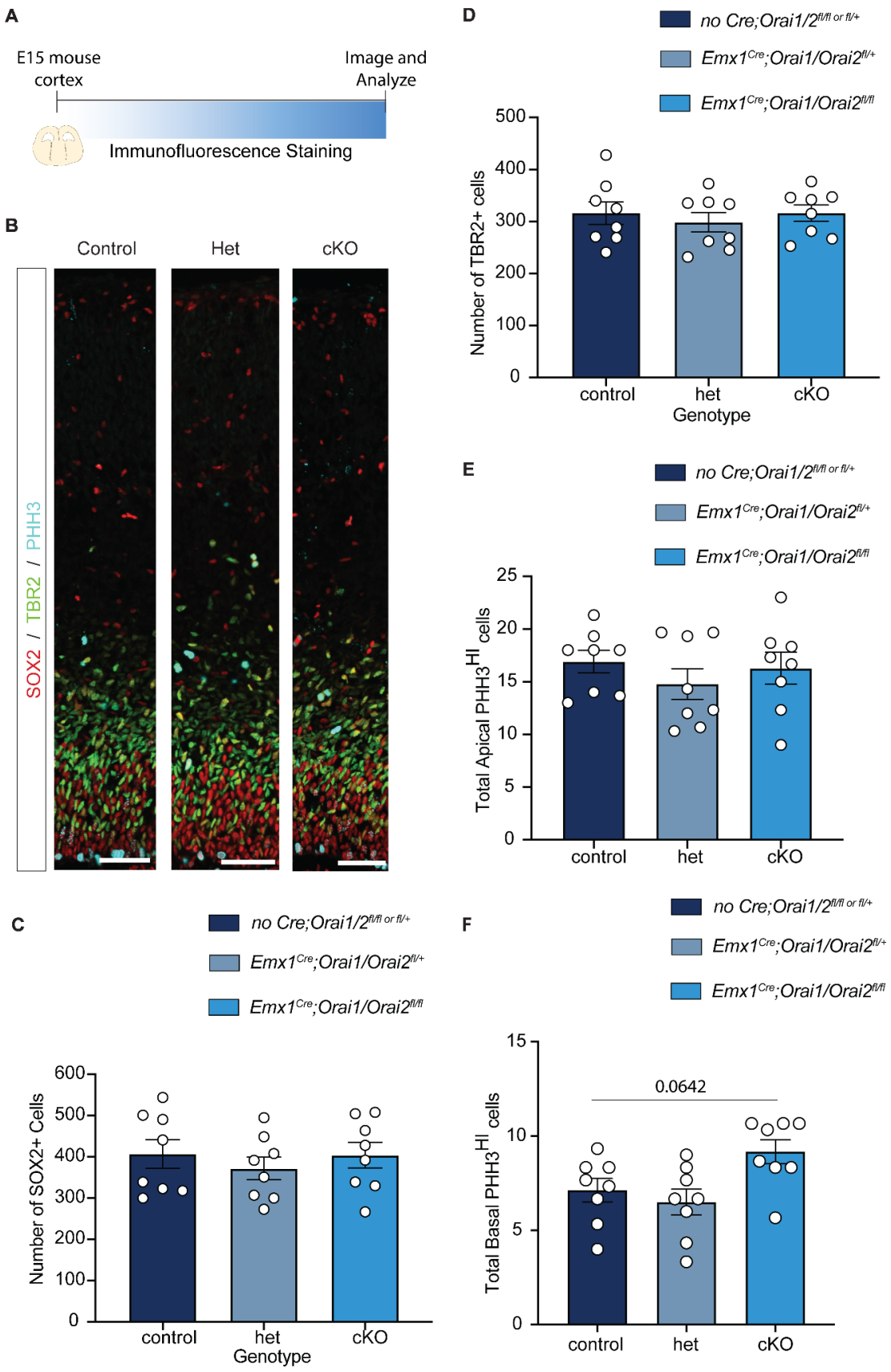
A
E15 mouse cortex
Image and Analyze
Immunofluorescence Staining
B
Control
Het
cKO
SOX2 / TBR2 / PHH3
C
no Cre;Orai1/2fl/fl or fl/+
Emx1Cre;Orai1/Orai2fl/+
Emx1Cre;Orai1/Orai2fl/fl
Number of SOX2+ Cells
600
500
400
300
200
100
0
control
het
cKO
Genotype
D
no Cre;Orai1/2fl/fl or fl/+
Emx1Cre;Orai1/Orai2fl/+
Emx1Cre;Orai1/Orai2fl/fl
Number of TBR2+ cells
500
400
300
200
100
0
control
het
cKO
Genotype
E
no Cre;Orai1/2fl/fl or fl/+
Emx1Cre;Orai1/Orai2fl/+
Emx1Cre;Orai1/Orai2fl/fl
Total Apical PHH3HI cells
25
20
15
10
5
0
control
het
cKO
F
no Cre;Orai1/2fl/fl or fl/+
Emx1Cre;Orai1/Orai2fl/+
Emx1Cre;Orai1/Orai2fl/fl
Total Basal PHH3HI cells
0.0642
15
10
5
0
control
het
cKO

Figure 3.3.4 – The total number of RGCs and IPCs in cortices from control mice and *Emx1^{Cre};Orai1/2^{fl}* heterozygous and conditional knockout mutants is unchanged.
A) Schematic illustrating experimental timeline. E15 mouse brains were harvested for immunofluorescence analysis. Immunofluorescence staining was performed to label RGCs with SOX2, IPCs with TBR2, and mitotic cells with □hosphor-histone H3 (PHH3).
B) Representative coronal sections through E15 mouse cortices immunostained using antibodies against SOX2 (red), TBR2 (green), and PHH3 (teal). Scale bar, 50 μm.
C-D) No differences in the total number of SOX2- **I** or TBR2 **(D)-**positive cells in region-matched 100μm windows spanning the cerebral wall of control mice and *Emx1^{Cre};Orai1/2^{fl}* heterozygous and cKO mutants. (data represent cells from region-matched serial sections; n= 8 brains per condition; mean ± SEM; one-way ANOVA, Dunnet's multiple comparisons test).
E) No differences in the number of apical PHH3 cells in region-matched 250μm windows spanning the cerebral wall of control mice and *Emx1^{Cre};Orai1/2^{fl}* heterozygous and cKO mutants. (data represent cells from region-matched serial sections; n= 8 brains per condition; mean ± SEM; one-way ANOVA, Dunnet's multiple comparisons test).
F) There is a modest increase in the number of basal PHH3+ cells in region-matched 250μm windows spanning the cerebral wall of control mice and *Emx1^{Cre};Orai1/2$^{fl/fl}$* homozygous mutants. (data represent cells from region-matched serial sections; n= 8 brains per condition; mean ± SEM; one-way ANOVA, Dunnet's multiple comparisons test).
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To determine whether there are any major alterations in the relative abundance of populations of NSPCs upon conditional deletion of *Orai1* and *Orai2*, we also quantified region-matched sections from E15 *Emx1^{Cre};Orai1/2^{fl}* animals for the total number of SOX2-positive RGCs and TBR2-positive IPCs in the VZ/SVZ across all three genotypes. We found no significant differences in the total number of SOX2- and TBR2-expressing cells in our region matched sections; however, we do not know whether the relative proportion of each cell type is different when taking into account the total number of cells in the VZ/SVZ. Completing this analysis at E15 and following it up with a similar analysis at later time points will thus be important. We also counted □hosphor-histone H3 (PHH3)-expressing cells in region-matched sections from E15 *Emx1^{Cre};Orai1/2^{fl}* cortices, restricting our analysis to cells expressing high, strongly nuclear PHH3 (PHH3^{hi}), which corresponds to cells in M-phase. While there was no apparent difference in the total number of PHH3^{hi} cells in the VZ/SVZ, when we split the mitotic cells into those that were localized apically (corresponding to the apical VZ/ventricular wall) or basally (corresponding to

abventricular mitoses in the basal VZ/SVZ), we found a trend towards increased basal PHH3^{hi} cells in *Emx1^{Cre};Orai1/2$^{fl/fl}$* mutant animals. Whether this corresponds to a chance in the fraction of total cells undergoing mitosis remains unclear.

While pharmacological inhibition of SOCE and *in utero* electroporation of *Stim2* splice variants with antagonistic effects on SOCE both altered NSPC proliferation, our surprising results thus far in the *Emx1^{Cre};Orai1/2^{fl}* animals suggest that dual deletion of *Orai1/2* largely does not affect NSPC proliferation. One potential explanation for this discrepancy is that *Orai1/2*-mediated calcium rises in different cell populations may have opposing effects on the cell cycle. While analyzing the total number of proliferative cells across the entire cerebral wall does not reveal any gross differences in proliferation between control and *Emx1^{Cre};Orai1/2^{fl}* heterozygous and homozygous mutant cortices, our PHH3 quantification, which separately assessed basally and apically localized mitotic NSPC populations, reveals that analyzing proliferation in RGCs and IPCs separately may be required to fully capture the effects of *Orai1/2* inactivation. As discussed earlier, in the context of depolarization-induced calcium influx, GABA was previously shown to have opposing effects on VZ and SVZ progenitor proliferation (Haydar et al., 2000). Thus, moving forward, it will be important to separately analyze the consequences of *Orai1/2* deletion on RGCs and IPCs. RGCs have been shown to radially propagate calcium signals along their processes to promote bidirectional communication with newborn neurons (Rash et al., 2016) It should be noted here that *Emx1^{Cre}* mediates inactivation of *Orai1/2* across the entire RGC lineage (spanning RGCs, IPCs and neurons and glia). Thus, it will likely be difficult to completely isolate the effects of *Orai1/2* loss of function to the radial glial population itself, as the RGCs may be receiving signals from *Orai1/2* loss of function neurons.

Another explanation for the discrepancy between our pharmacological manipulations and *Stim2* variant electroporations that suggested that SOCE may promote proliferation and our results in *Emx1*^{Cre}*;Orai1/2*^{fl} mutants may be that *Orai1* and *Orai2* have opposing effects on the cell cycle that can not be appreciated in a double mutant. Spatially restricted microdomains around the mouth of ORAI channels determine the activation of downstream effector proteins, and thus, it is possible that calcium entry through *Orai1* and *Orai2* have distinct effects on NSPC biology depending on their relative abundance in NSPC subpopulations. Indeed, recent studies have shown that the ORAI calcium channels signal to different downstream effectors (Yoast, Emrich, Zhang, et al., 2020). Moreover, calcium entry via ORAI2 and ORAI3 has been shown to oppose ORAI1-dependent NFATc2/c3 nuclear translocation (Emrich et al., 2021; Yoast, Emrich, & Trebak, 2020; Yoast, Emrich, Zhang, et al., 2020), further supporting the idea that calcium entry through different ORAI homologs may produce diverse downstream effects. Interestingly, in the immune system, deletion of *Orai2* alone enhances SOCE, whereas dual *Orai1* and *Orai2* deletion completely abolishes SOCE, pointing to a complex relationship between the *Orais* and the possible formation of heteromeric channels (Vaeth et al., 2017). Indeed, the cytosolic calcium dynamics induced by influx through different ORAI homologs have distinct properties (e.g., differences in the amplitude of cytosolic calcium elevations), and the existence of heteromeric channels further diversifies the characteristics of ORAI-mediated calcium entry. By way of example, maximal store depletion preferentially activates ORAI1 over the other ORAI homologs. Calcium entry through ORAI1 homomeric channels yields sustained plateaus in cytosolic calcium concentrations. In contrast, ORAI2 and ORAI3 have been shown to participate in oscillatory calcium responses, while ORAI1 is dispensable for maintaining calcium oscillations (Yoast, Emrich, Zhang, et al., 2020). Different characteristics of calcium oscillations can differentially regulate downstream, calcium-sensitive

signaling cascades (Dolmetsch et al. 1998). Taken together, the importance of analyzing the effects of *Orai1* and *Orai2* single mutants on cortical development is clear. While it has been challenging for us to generate single mutants thus far from our double *Orai* mutants, as *Orai1* and *Orai2* are linked genes in the mouse and are largely inherited together, we are in the process of generating single mutant animals for our studies.

Finally, it is possible that SOCE does not contribute to NSPC proliferation, and that our pharmacological studies and *Stim2* variant electroporations perturb other regulators of NSPC biology. The IP3 receptor represents one of the molecules that lies at the intersection of STIM2 interactors and 2-APB pharmacology (Ahmad et al., 2022; Missiaen et al., 2001; Prakriya & Lewis, 2001). Indeed, untangling the developmental effects of IP3-dependent signaling and SOCE remains a tricky endeavor. SOCE is largely induced by activation of cell surface receptors that initiate Phospholipase C (PLC) signaling, which then leads to the generation of IP3. IP3 binds to and activates the IP3 receptor to allow calcium efflux from the ER into the cytosol. As sequential upstream stimuli continue to deplete ER calcium stores, the STIM ER calcium sensors activate SOCE to allow calcium influx via ORAI channels from the extracellular space (Clapham, 2007). IP3-induced calcium efflux from the ER is thus mechanistically tied to store depletion and SOCE, and many pharmacological agents that inhibit SOCE also modulate IP3-dependent signaling. Moving forward, it will be important to study the effects of knocking out the IP3 receptor in the cells of the developing cortex, in order to more fully understand how ER store-dependent IP3 calcium rises modulate NSPC behavior.

3.4 – Methods

3.4.1 – Animals

All mouse experiments were conducted in adherence to UCSF Institutional Animal Care and Use Committee (IACUC) standards and National Institutes of Health guidelines. Timed- pregnant wild type Swiss Webster mice were purchased from Charles River Labs (Stock #024). *Orai1*$^{fl/fl}$*;Orai2*$^{fl/fl}$ double-floxed mice were obtained from Dr. Regina Clemens (Orai1$^{fl/fl}$ was obtained from Amgen through Dr. Regina Clemens, Washington University, St. Louis; Orai2$^{fl/fl}$ (Dr. Regina Clemens, Washington University, St. Louis) mice were provided by Dr. Regina Clemens) To generate conditional forebrain-specific *Orai1;Orai2* knockout mice, *Orai1*$^{fl/fl}$*;Orai2*$^{fl/fl}$ double-floxed mice (Grimes et al., 2020) were mated to *Emx1*^{Cre} mice (JAX Stock No. 005628) (Gorski et al., 2002). To generate timed-pregnant mutant mice, the presence of the vaginal plug was labeled embryonic day 0.5 (E0.5). Genotyping of *Emx1*^{Cre};*Orai*^{fl} animals for colony maintenance was performed using tissue obtained from ear notches. The genotype of experimental mice was confirmed using liver tissue. For birthdating studies, pregnant dams received a single intraperitoneal injection of BrdU (5-bromo-2'-deoxyuridine/BrdU: Sigma Aldrich, B5002-1G) at 50mg/kg.

Genotyping primers were as follows:

1) ***Emx1*^{Cre} *genotyping (Joyner Lab; 300bp amplicon)***

Emx1^{Cre} forward: TAA AGA TAT CTC ACG TAC TGA CGG TG

Emx1^{Cre} reverse: TCT CTG ACC AGA GTC ATC CTT AGC

2) ***Orai1 genotyping (Taconic; 390bp cKO amplicon, 390+260bp heterozygous amplicon, WT=260bp amplicon)***

Orai1 forward: GAA ATG GCT CGG GGA CAA AAC ACT A

Orai1 reverse: GCC ATT TCT GGT CTT CTG GAG ACT CTG

3) ***Orai2 genotyping (Taconic; 256bp WT amplicon, 376bp cKO amplicon)***

Orai2 forward: ACTAAGGCTGAGGCTAAGTCAGG

Orai2 reverse: TGTCTGTTTCCAGAGGAAGGC

3.4.2 – Mouse cortical cell cultures

Embryonic mouse brains were initially dissected in cold HBSS and cortices were carefully dissected after removal of meninges. Tissue was digested in papain (Worthington, LS003119) for 7 minutes at 37C and then incubated in Trypsin Inhibitor (Sigma Aldrich, SIAL-T9253-1G) at room temperature. Cortices were dissociated into single cell suspensions in HBSS containing Dnase. Cells were pelleted and washed with HBSS and subsequently resuspended in NSPC media (Neurobasal media (ThermoFisher Scientific, 21103-049) containing B27 (ThermoFisher Scientific, 12587-010), Glutamax (ThermoFisher Scientific, 35050061) and penicillin-streptomycin (ThermoFIsher Scientific, 15140-122). Cells were plated on poly-ornithine (Sigma P4957) and laminin (Sigma L2020) coated cell culture plates or coverslips. NSPC cultures were supplemented with 20ng/mL of EGF (Peprotech, AF-100-15) and FGF2 (Peprotech, AF-100-18B).

3.4.3 – Calcium Imaging

Mouse or human cell cultures were transduced with a lentivirus expressing a CAG driven jGCaMP7f-T2A-tdTomato on the day the cells were plated. Three days after cells were transduced, cells were used for calcium imaging. Cells were imaged on an upright epifluorescence microscope (Olympus BX51WI) using a water immersion 40X objective with a Lamda 421 light source. For SOCE experiments, cells were first bathed in Tyrode's solution containing 2mM CaCl2 (129mM NaCl, 5mM KCl, 2mM CaCl2, 1mM MgCl2, 30mM Glucose, 25mM HEPES). Cells were then bathed in 0mM CaCl2 containing Tyrode's (129mM NaCl, 5mM KCl, 0mM CaCl2, 3mM MgCl2, 30mM Glucose, 25mM HEPES) and subsequently perfused with 0mM CaCl2 Tyordes containing 1uM Thapsigargin. When cytosolic calcium returned to baseline levels, cells were perfused with 2mM CaCl2 Tyrodes to elicit SOCE. The same paradigm was used for purinergic signaling calcium imaging experiments, with the exception that 100uM MesADP was applied in 0mM CaCl2 Tyrode's in lieu of TG. Metamorph Software was used to collect calcium imaging time-lapse fluorescence images in the GFP channel. Individual cells were either outlined as regions of interest using FIJI (Schindelin et al., 2012) or Cell Profiler (Carpenter et al., 2006) and mean intensity measurements were extracted and plotted using a custom Python script. For SOCE calcium imaging on WT embryonic mouse or primary human NSPCs, responding cells were defined as those that responded to both Thapsigargin (TG) and SOCE. A TG responder was defined as having a calcium rise greater than baseline levels that lasted for 50 seconds in the window of duration of TG application. A SOCE rise was defined as being greater in amplitude than baseline levels. Responders from SOCE imaging of $Emx1^{Cre}$;$Orai^{fl}$ animals were defined as those that had only a TG rise.

3.4.4 – Primary human cortical slices and dissociated cell cultures

Under protocols approved by the UCSF Institutional Review Board (IRB) and Human Gamete, Embryo, and Stem Cell Research Committee (GESCR), we obtained de-identified embryonic human cortical tissue with prior informed consent. To make slice cultures of primary tissue, we embedded samples in low melting temperature agarose and vibratome sectioned them in ACSF (125mM NaCl, 2.5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 1.25mM NaH_2PO_4, 25mM $NaHCO_3$, 25mM Glucose). ACSF was bubbled with 95% O_2, 5% CO_2 immediately prior to vibratome sectioning. 300 micron slices were cultured on Millicell culture inserts (Millipore PICM03050) in the air/liquid interface in slice culture media containing 31% HBSS (Hanks' Balanced Salt solution without Calcium and Magnesium), 60% Basal Media Eagle, 5% FBS, 1% glucose, 1% N2 Supplement (Thermofisher Scientific, 17502-048), 1% Glutamax (Thermofisher Scientific, 35050061) and 1% Pennicillin-Streptomycin. Slices were maintained in culture for a maximum of 4 days. For experiments in which slices were dosed with 2-APB, slices were vibratome sectioned and cultured in slice culture media for 24-48hours. 2-APB was subsequently applied to slice culture media for 24 hours. Two hours prior to ethanol fixation for cell cycle analysis, slices were pulsed with 10uM BrdU. To generate dissociated cell cultures of primary human tissue, we first subdissected germinal zones and dissociated them in papain (Worthington, LS003119) for 15 minutes at 37C. We then incubated the tissue in Trypsin Inhibitor (Sigma Aldrich, SIAL-T9253-1G) at room temperature, after which cortices were dissociated into single cell suspensions in human NSPC media (DMEM/F-12 with N2, B27, 1% Glutamax, 1% penicillin-streptomycin and sodium pyruvate (ThermoFisher Scientific, 11360-070) containing Dnase (Sigma Aldrich, D4513). Cells were pelleted, washed with NSPC media twice, and plated on Matrigel (Corning

354234) pre-coated glass coverslips in NSPC media supplemented with 20ng/mL FGF2 (Peprotech, AF-100-18B) and EGF (Peprotech, AF-100-15).

3.4.5 – Immunohistochemistry (Tissue preparation, Immunofluorescence staining)

Embryonic mouse brains were dissected in cold HBSS (ThermoFisher, 14175145) and fixed for 30 minutes in 4% PFA in PBS at 4C. Brains were washed in PBS, placed in 15% sucrose for 1hr and then subsequently placed in 30% sucrose until they sank. Prior to cryosectioning, brains were equilibrated for an hour in Optimal Cutting Temperature (OCT Tissue-Tek, 4583) and then frozen in fresh OCT on dry ice. Sections were cut on a cryostat at 16μm and kept at -80C until immunostaining.

For immunostaining, frozen sections were equilibrated to room temperature, washed with PBS and blocked for one hour in blocking solution (10% normal goat or donkey serum, 0.3% TritonX-100 in PBS). Overnight primary antibody incubation was performed in 2% serum (normal goat or donkey serum) and 0.1% TritonX-100 in PBS at 4C. The next day, sections were washed at least three times for five minutes with PBS, and Alexa secondary antibodies (ThermoFisher Scientific) were applied in 2% serum (normal goat or donkey serum) and 0.1% TritonX-100 in PBS for one hour. Sections were washed at least three times for five minutes. Nuclei were stained using 1:10000 Hoescht 33258 (Invitrogen), and slides were mounted and coverslipped with Fluorogel (EMS, 17985-10). For BrdU staining, primary and secondary antibody staining for all other antigens was first performed, then sections were post-fixed with cold PFA (4C) for 10min, and subjected to 2N HCl antigen retrieval for 30min at 37C. Incubation in 0.1M sodium borate for 10min was performed to neutralize HCl antigen retrieval. Subsequently, sections were blocked for one hour in blocking solution and incubated in BrdU primary antibody in 2% serum (normal goat

or donkey serum) and 0.1% TritonX-100 in PBS at 4C overnight. The subsequent day, sections were washed in PBS and were incubated in secondary antibodies for BrdU. Antibodies used: TBR2/Eomes (Rat, Thermo Fisher, 14-4875-82), SOX2 (Goat, Santa Cruz, sc17320), PH3 (Rabbit, Millipore Sigma, 160189, lot: 3031618); and GFP (Chicken, Abcam, ab13970, lots: GR3190550-13, GR3190550-8, GR236651-27) ; Ki67 (Mouse, BD Pharmingen, 550609); BrdU (Rat, abcam, ab6326, lots: GR3173537-9, GR3173537-10)

3.4.6 – Microscopy

Images were acquired on a Zeiss Axioscope Imager.M2 using a 20X objective, a Leica SP8 Laser Scanning Confocal Microscope with White-Light Laser using a 25X objective, or the Leica Stellaris Laser Scanning Confocal Microscope using a 20X objective. Images were tiled and stitched when necessary to capture the entire span of the counting region. For confocal images, two z-stacks were taken 2-3uM apart and maximum intensity projections were used of confocal images for quantitative analysis. Images in the figures were rotated, cropped to display relevant cortical regions and were post-processed with brightness contrast modifications (gamma was unchanged). For quantitative analysis, cells were counted with the investigator blinded to genotype or electroporated construct, using Imaris Image Analysis Software (Oxford Instruments) or the ImageJ (cite: Public Domain, BSD-2) Cell Counter application. Cells from 2-4 region-matched sections from 3-8 animals were counted for each experiment, and results from at least two litters were used for each experimental group. Prism 9 (Graphpad) was used for plotting results and performing statistical analyses. Detailed reporting of statistics and sample size numbers is included with respective figure legends.

3.4.7 – Flow Cytometry

Fluorescence activated cell sorting (FACS) for "Flashtag" injections

All "Flashtag" (FT) injections were performed at E13.5. At different time points after "Flashtag" (FT) injection (1 hour, 10 hours, 24 hours, and 4 days, corresponding to the labeling of RGCs, IPCs, newly born immature neurons and postmitotic neurons, respectively), dams were euthanized and embryonic brains were dissected in HBSS (4°C). At 1 hour and 10 hours after FT, a 20uL pipette tip was used to flush the ventricles of dissected brains in order to dilute any residual FT solution. Brains were visualized under a CFSE excitation light source in order to verify whether all brains were appropriately injected. Brains in which FT was not injected optimally were discarded at this step. After careful removal of the meninges, individual cortical hemispheres were dissected and separately processed in downstream steps. Cortices were first enzymatically digested for 7 min at 37°C using papain (Worthington, LS003119). Following incubation in Trypsin Inhibitor (Sigma Aldrich, SIAL-T9253-1G), cortices were dissociated into single cell suspensions in HBSS (4°C) with DNAse (Sigma). Single cell suspensions from each cortical hemisphere was kept separate and filtered prior to sorting on a BD FACS Aria. Dead cells, which were labeled using Sytox Red (Life Technologies, S34859), and doublets were excluded, and the top 5-10% of FT-expressing cells were then collected in Buffer RLT (from the Qiagen Rneasy Micro Kit, 74004) for subsequent RNA extraction. FACS gates were defined as detailed in Govindan et al., 2018.

Flow cytometry for cell cycle analysis

Cell cycle analysis was performed on *in vitro* dissociated cell cultures, primary human slice cultures and mouse cortices. *In vitro* cultures were detached from cell culture dishes using TrypLe (Invitrogen), washed, and subsequently fixed in 75% ethanol in PBS at -20C and subsequently stained for cell cycle analysis (below). Germinal zones from primary human slice cultures were subdissected, while mouse cortices were dissected in HBSS (4°C). Slice cultures and mouse cortices were digested in papain for 10 minutes at 37°C. Subsequently, papain was removed from the tissue and cells were immersed in trypsin inhibitor. Tissue was then dissociated into single cell suspensions in HBSS (4°C), washed twice, fixed with 75% ethanol at least overnight at -20°C and subsequently stained for cell cycle analysis.

To stain and prepare cells for cell cycle analysis, fixed cells were pelleted and subsequently resuspended in PBS for 5 minutes. Cells were then pelleted again and resuspended in 0.5% BSA in PBS. Once the cells were rehydrated, cells were counted. One million cells per sample were subsequently stained for BrdU. These cells were pelleted, and denatured using 2M HCl with 0.5% TritonX-100 for 20 minutes. Cells were then pelleted and neutralized for 2 minutes in 0.1M sodium borate. Neutralization buffer was washed off, and cells were blocked in 0.5% BSA in PBS prior to primary antibody incubation. The primary antibody, an Alexa Fluor 647-conjugated antibody directed against BrdU (BD Pharmingen, 560209, 1:100), was diluted in 0.5% BSA with 0.5% Tween-20 in PBS. Cells were resuspended in 100ul of antibody solution and were kept overnight at 4°C. The next day, cells were pelleted, washed in PBS with 0.5% BSA and subjected to propidium iodide (PI) staining. For PI staining, cells were resuspended in a staining buffer containing 1X Rnase A (ThermoScientific, EN0531), propidium iodide (Life Technologies, P1304MP) and PBS for 30 minutes at room temperature. Stained cells were analyzed on a BD

FACSCanto2 (BD Pharmingen). Percentage of cells from each stage of the cell cycle was calculated with either FlowJo or BD FACS Diva software.

3.4.8 – "Flashtag" injections and in utero electroporations

All surgical instruments were sterilized, glass micropipettes were prepared, and sterile saline was pre-warmed prior to *in utero* surgeries. Glass micropipettes were pulled on a Sutter Instrument P97 Micropipette puller and were beveled such that they had a slanted, sharp tip 30-70μM in outer diameter. For "Flashtag" (FT) *in utero* injections, a FT working solution was also prepared pre-operatively as follows: 8μl of DMSO and 1μl of 0.01% Fast Green were added to one vial of CellTrace CFSE (Thermo Fisher, C34554) (as in Govindan et al., 2018). For *in utero* electroporations, overexpression plasmids were diluted to 1μg/μl while shRNA constructs were prepared at 2μg/μl.

E13-E14 timed-pregnant Swiss Webster dams (E14, Charles River Labs) were deeply anesthetized using isofluorane (2-4% to effect). Pre-operative multimodal analgesia (0.1mg/kg Buprenorphine hydrochloride and 5mg/kg Carprofen) was administered subcutaneously prior to incision. Throughout the surgical procedure, aseptic technique was employed. After shaving and sterilizing the abdomen of pregnant dams, a midline incision was used to expose the uterine horns, which were gently placed onto sterile gauze pads. The uterine horns were continuously bathed in pre-warmed, sterile saline throughout the duration of the surgery. For "Flashtag" injections, approximately 0.5μl of "Flashtag" was injected into the lateral ventricle of individual embryos using a pulled and finely beveled tip. At this point, for *in utero* electroporations, 1μl of plasmid DNA was injected into the lateral ventricle of individual embryos. Paddle electrodes were gently placed on either side of the embyro's head, on top of the uterine horns. The positive paddle was

placed on top of the cortex to guide the DNA into the RGCs lining the ventricles. Using a BTX electroporator, five 50ms pulses of 38V were delivered to each embryo injected with plasmid DNA. After FT or DNA injections were completed, the uterine horns were returned to the abdominal cavity and once again moistened with pre-warmed saline. The incision was closed in two layers, accompanied by local application of lidocaine prior to final closure. Animals were allowed to recover in heated recovery cages until alert and ambulating freely, at which point they were returned to their home cages.

3.4.9 – qRT-PCR

RNA was extracted using the Qiagen Rneasy Micro kit (for small cell numbers post-FACS) or the Rneasy Mini kit (for larger quantities of tissue). DNAse digestion was performed on column when using the Rneasy Micro kit, while it was performed after elution when RNA was isolated with the Rneasy Mini kit. RNA concentration was subsequently measured using the Agilent Bioanalyzer (using the Agilent RNA 6000 Pico Kit) or a nanodrop dependending on concentration. cDNA was subsequently generated by reverse transcription using Superscript III, (Invitrogen, 11752050). Quantitative real-time PCR (qRT-PCR) with specific primers directed to genes of interest was performed using SYBR GREEN (Roche, 4707516001) to assess gene expression. Technical duplicates or triplicates were performed for qRT-PCR reactions. Ct values were normalized to *Actb* or *Gapdh* and Transcript abundance was calculated using the following formula:

$(E_{\text{target}})^{-\text{Ct}\,(target)} / (E_{Gapdh \text{ or } Actb})^{-\text{Ct}\,(Gapdh\ or\ Actb)}$

mouse *Gapdh* forward: 5’ AGGTCGGTGTGAACGGATTTG 3’

mouse *Gapdh* reverse: 5’ TGTAGACCATGTAGTTGAGGTCA 3’

mouse *Actb* forward: 5’ TGACGTTGACATCCGTAAAG 3’

mouse *Actb* reverse: 5’ GAGGAGCAATGATCTTGATCT 3’

mouse *Ki67* forward: 5’ AATCCAACTCAAGTAAACGGGG 3’

mouse *Ki67* reverse: 5’ TTGGCTTGCTTCCATCCTCA 3’

mouse *Pax6* forward: 5’ TACCAGTGTCTACCAGCCAAT 3’

mouse *Pax6* reverse: 5’ TGCACGAGTATGAGGAGGTCT 3’

mouse *Tbr2* forward: 5’ AGCTAAAGATCGACCATAACC 3’

mouse *Tbr2* reverse: 5’ CACCGTTATAATATCGGGCT 3’

mouse *Dcx* forward: 5’ CATTTTGACGAACGAGACAAAGC 3’

mouse *Dcx* reverse: 5’ TGGAAGTCCATTCATCCGTGA 3’

mouse *Map2* forward: 5’ CTCATTCGCTGAGCCTTTAGAC 3’

mouse *Map2* reverse: 5’ ACTGGAGGCAACTTTTCTCCT 3’

mouse *Orai1* forward: 5’ GTTCTTACCTCTCAAGAGG 3’

mouse *Orai1* reverse: 5’ AACCATGATGGCGGTGGA 3’

mouse *Orai2* forward: 5’ TGAGTGCAGAGCTCAATGT 3’

mouse *Orai2* reverse 5’ TGGTTAGACGTGACGAGTT 3’

mouse *Orai3* forward 5’ CATATTGAAGCCGTGAGCA 3’

mouse *Orai3* reverse 5’ GAACAACTTCAGCCAGGAA 3’

mouse *Stim1* forward 5’ GAT TTG ACC CAT TCC GAT TC 3’

mouse *Stim1* reverse 5’ CAT TGG AAG GCA TGG CAT T 3’

mouse *Stim2α* forward 5’GCCATCGCTAAGGACGAGGCA 3’

mouse *Stim2β* forward 5’TCGCTGCCTCCTATCTCCTG 3’

mouse *Stim2α/β* reverse 5’ CTACTTCGTCCAGGGAGGAGC 3’

3.4.10 – Data analysis and statistics

All image quantifications were performed blinded to genotype or experimental condition. Statistical significance was assumed to be $p<0.05$. Sample sizes were determined based on previous studies, power calculations using pilot experiments or our own experience to estimate effect size of an assay. For cell cycle experiments, flow cytometry analyzed samples were discarded only if BrdU staining was poor or if cell numbers were < 5000 cells. For calcium imaging experiments, samples were discarded if baselines were steadily decreasing over the course of the entire experiment, or if there was an artifact during imaging (i.e. sample focus or movement artifact). Prism software (GraphPad) was used to perform all statistical analyses. Statistical tests used are noted in figure legends.

Chapter 4 – Conclusions, Ongoing Studies and Future Directions

This chapter includes discussion of some of the experiments I performed towards a manuscript being finalized for submission:

Petrova R, **Arjun McKinney A**, Wu B, Torres T, Hamid S, Delgado RN, Ki C, Su Z, Qui L, Pippin H, Nowakowski TJ, Ellegood J, Lim DA, Graef I, Darmanis S & Panagiotakos G. "DYRK1A Kinase Regulates Cortical Development via NFAT-mediated Cell-specific Modulation of Calcium Signaling."

4.1 – Calcium entry in the developing cortex

This dissertation is centered around calcium signaling pathways that are active in NSPCs of the developing cortex. My primary thesis work, expanded upon in Chapter 3, has been aimed at investigating functions of SOCE during embryonic cortical development. To date, we have defined cell type-specific expression of SOCE molecules in the developing cortex and shown, using calcium imaging, that SOCE can be induced in both mouse and human primary cortical NSPCs. Intriguingly, we found that ATP signaling is a potential upstream activator of SOCE in NSPCs and that pharmacological inhibition of SOCE suppresses proliferation of primary mouse and human cortical cells. We also found that expression of two *Stim2* variants (with known antagonistic effects on SOCE amplitude) bidirectionally regulate NSPC cell cycle exit *in vivo*. While our preliminary analyses suggest that dual conditional deletion of *Orai1/2* does not grossly alter proliferation across the entire NSPC compartment in the embryonic cortex, it will be necessary moving forward to dissect cell type-specific differences in proliferation in the context of

conditional *Orai1/2* inactivation. We also plan to carefully phenotype mice lacking *Orai1* or *Orai2* individually and to begin to probe the contribution of store-dependent IP3 signaling to NSPC biology.

Much of the work in this thesis is concerned with calcium entry and potential upstream mechanisms by which calcium enters the cell. However, much remains to be elucidated about downstream signaling pathways that transduce calcium signals into changes in gene expression in the developing cortex. In physiological settings, store depletion triggers a complex interplay between the STIM sensors and ORAI channels, ultimately resulting in calcium trickling through ORAI channels at high specificity and low unitary conductance. This calcium influx can last up to hours, and its characteristics are ideal for creating spatially restricted calcium microdomains that can serve as signaling hubs to the nucleus, selectively activating distinct transcription factor cascades. Indeed, different properties of SOCE-dependent calcium elevations can recruit specific transcription factors to influence cellular behavior (Dolmetsch et al., 1997, 1998). Multiple transcription factors have been shown to respond to calcium influx via SOCE in different contexts and cell types, including NFAT, NFκB, CREB and Sp4 (Avila-Medina et al., 2018; Berridge et al., 2000; Berry et al., 2018; Gwack et al., 2007; Kozak & Putney, 2017; Lalonde et al., 2014; Pulver et al., 2004; Yeh & Parekh, n.d.). Each of these transcription factor families represents a potential effector of SOCE that can be investigated in future studies of lineage progression in the embryonic cortex. To define the mechanisms and molecules by which calcium entry activates specific transcriptional programs at different times or cell states, we must decode how calcium transients carry information in their spatiotemporal properties (including amplitude, frequency and duration of the calcium rise). One way to approach this is to perform experiments in engineered NSPCs in which intracellular calcium levels are under optogenetic control (Cheng et al., 2021; L.

He et al., 2015, 2021; N. T. Nguyen, He, et al., 2018; N. T. Nguyen, Ma, et al., 2018). Indeed, expression of optogenetic constructs in cells of the developing cortex would allow us to tune the spatiotemporal properties of cytosolic calcium elevations in NSPCs, thus allowing us to interrogate how different patterns of calcium influx regulate downstream signaling. Pairing this approach with immunofluorescence analysis, as well as biochemical and luciferase assays, to read out the activity of downstream transcription factors is one way in which we can further elucidate calcium signaling specificity. Finally, to fully understand how calcium induces changes in cellular behaviors in the developing cortex, it will be crucial to determine how this regulation differs at a cell type-specific level. Altogether, these efforts would begin to tackle the question that lies at the core of developmental neurobiology: how do extrinsic signals influence genetic programs inside different cell types to drive changes in cellular behavior?

4.1.1 Intersection of different modes of calcium entry in the developing cortex

How different mechanisms of calcium influx work in conjunction with each other to orchestrate calcium signaling remains poorly understood. In the developing cortex, for example, two primary mechanisms of calcium entry are driven by voltage gated calcium channels (VGCCs) and CRAC channels, in response to electrical signals or depletion of intracellular stores of calcium, respectively. While relatively little is known about the regulation of these two calcium entry mechanisms in the context of immature cortical progenitors, studies of postnatal neurons and vascular smooth muscle cells have suggested that these calcium entry pathways negatively regulate each other (C. Y. Park et al., 2010; Y. Wang et al., 2010). Via its cytosolic CRAC-activating domain (CAD), which normally activates ORAI channels, STIM1 was shown to inhibit the L-type channel (LTC) Cav1.2 through an interaction with its intracellular C-terminus that results in

channel internalization and degradation, and thus downregulation of LTC currents (C. Y. Park et al., 2010; Y. Wang et al., 2010). Emerging evidence further extends STIM1's role as a VGCC inhibitor to T-type channels, suggesting that STIMs may negatively regulate VGCCs more broadly across subtypes (N. Nguyen et al., 2013). In a cardiomyocyte-derived cell line, knockdown of STIM1 increased T-type VGCC conductances, and co-immunoprecipitation experiments suggest direct interactions between STIM1 and the T-type VGCC $Ca_v3.1$ (N. Nguyen et al., 2013). Conversely, membrane potential may influence calcium influx into the cell via SOCE, as depolarization prevents calcium entry through Orai channels while hyperpolarization promotes calcium entry (Bakowski & Parekh, 2000; Bird & Putney, n.d.). This reciprocal regulation of voltage-dependent and ER store-dependent calcium entry suggests another layer of cell state-specific tuning of calcium signaling that is necessary to consider when interrogating calcium dynamics in developing cell populations. As *Stim2* is more highly expressed in the embryonic cortex than *Stim1*, one interesting lingering question is whether the *Stim2* CAD is also able to inhibit voltage-gated calcium channels, which are increasingly being shown to play important developmental roles during corticogenesis. Another interesting idea to consider is whether populations of patients with *Stim1* loss-of-function mutations (Lacruz & Feske, 2015) display alterations in calcium influx through VGCCs and voltage-dependent calcium signaling. Finally, as ventricular zone progenitors become gradually hyperpolarized as development progresses (Vitali et al., 2018), it will be crucial to understand the complex interplay between membrane depolarization and calcium entry across RGC populations in embryonic time. As these studies begin to elucidate the intersection of different modes of calcium entry, it becomes increasingly apparent that to fully understand the regulation of calcium in developing cells we must systematically define the expression of ion channels and calcium-sensitive molecules (and their

splice isoforms) at a cell type-specific level. Such an effort will allow us to build a more complete understanding of how calcium signaling pathways work in conjunction with each to alter cellular behavior.

Indeed, inspired by growing evidence that alternative splicing is essential for normal cortical development and is deregulated in neurodevelopmental disease (Eom et al., 2013; Irimia et al., 2014; Leung et al., 2021; Patowary et al., n.d.; Quesnel-Vallières et al., 2016; Splawski et al., 2004; Vuong et al., 2016; X. Zhang et al., 2016), we have been pursuing a focused set of experiments aimed at characterizing full length isoforms of channels and proteins involved in calcium signaling in specific populations of developing cortical cells. It is clear that alternative splicing is an important regulator of calcium signaling, generating calcium channels and signaling proteins that are functionally distinct across different cell types or across developmental time (Lipscombe et al., 2013; Panagiotakos et al., 2019; Splawski et al., 2004, 2005; Tang et al., 2004, 2007, 2009). We have thus been isolating populations of embryonic RGCs, IPCs, immature migratory neurons and post-mitotic neurons using FlashTag in the embryonic mouse (**Figures 3.2.6-3.2.7**) for long-read, Single Molecule Real-time (SMRT) Isoform Sequencing (using the Pacific Biosciences Iso-Seq platform), with the goal of identifying cell type-specific splice isoform expression of ion channels, pumps and calcium signaling molecules. Our initial efforts multiplexed 8 samples (4 cell types, 2 replicates each) in a single flow cell and gave us a first glimpse of potential cell type-specific isoforms.

Tantalizingly, we have found that differential isoform utilization during RGC lineage progression includes ion channel subunits, such as *Gria2,* which encodes the Glutamate Ionotropic Receptor AMPA Type Subunit 2 (GluA2). Inclusion of GluA2 renders AMPARs calcium-impermeable (Sommer et al., 1991), and its expression has been shown to increase postnatally,

implying that AMPARs in the embryonic brain are likely to allow calcium influx (Kumar et al., 2002). In line with observations in the literature, our preliminary analysis on our long-read dataset confirms that total transcript levels of *Gria2* are low in RGCs but steadily increase across the lineage and are highest in the 4 day postmitotic neuronal population (Kumar et al., 2002). Consistent with the presence of calcium-permeable AMPARs in embryonic cortical cells, AMPA agonists induce calcium rises in proliferating NSPCs, while AMPA antagonists block glutamate-induced calcium rises in the cells of the cortical germinal zones (LoTurco et al., 1995; Maric et al., 2000). Moving forward, it will be crucial to interrogate the electrophysiological properties of the various *Gria2* splice variants, as well as their cell biological functions during cortical development. As mutations in *GRIA2* are reproducibly linked to disorders of developmental origin like autism spectrum disorder (ASD) and intellectual disability (ID), it will also be important to determine whether newly identified splice variants are differentially susceptible to disease-relevant mutations and/or whether their cell type-specific expression renders certain developing populations more vulnerable to the effects of disease-causing genetic variants (C Yuen et al., 2017; De Rubeis et al., 2014; Hackmann et al., 2013; Salpietro et al., 2019)

Importantly, limitations of sequencing depth in this initial paradigm made the identification of variants of low abundance transcripts (which include voltage-gated calcium channels) difficult, prompting us to complement these efforts with a targeted enrichment strategy using probes specific to a panel of ~50 calcium-associated genes. By specifically pulling-down transcripts from genes of interest, which include voltage gated calcium channels and the channels mediating SOCE, this targeted search will eschew sequencing depth problems and help us begin to identify the full isoform diversity of calcium-related genes. Ultimately, defining the cell type-specific expression patterns of the major calcium signaling genes and their splice variants will better enable us to

explain how calcium signaling is shaped by the intersection of different modes of calcium entry. As aberrant calcium signaling has been implicated in various neurodevelopmental disorders (see Table 1.1 in Chapter 2) (Arjun McKinney et al., 2022), our studies defining the molecules and mechanisms involved in transducing calcium signals during cortical development will provide essential insights into normal and dysfunctional corticogenesis.

4.2 – Which intracellular pathways transduce calcium signals in the developing brain?

4.2.1 Elucidating roles for the NFAT export kinase, DYRK1A, in the developing cortex

While the main portion of my thesis work interrogates calcium entry and its regulators of in developing cortical cells, much is unknown about how these mechanisms are linked to downstream pathways and transcription factors that transduce cytosolic calcium rises into gene expression changes in the embryonic cortex. In T cells, where SOCE represents one of the primary modes of calcium entry, calcium signaling via SOCE activates calcineurin, which promotes the nuclear import of NFATc proteins, where they bind DNA to alter gene expression (Gwack et al., 2007). As such, it is possible that SOCE activates NFAT signaling, long studied as a key signaling pathway in multiple developing tissues (Chang et al., 2004; Graef et al., 2001), in the developing cortex. VGCCs have also been shown to potently activate NFAT-dependent transcription in postnatal hippocampal neurons (Catterall, 2011; Graef et al., 1999; Murphy et al., 2019; Oliveria et al., 2007). As multiple modes of calcium entry likely converge on NFAT in the developing cortex, a major interest of our lab has centered around understanding how NFAT integrates these responses to drive cellular behaviors.

A significant portion of my graduate work involved contributions to a study (manuscript currently being finalized for submission, not included in this dissertation) interrogating the roles of the DYRK1A, a kinase that effectively antagonizes NFAT signaling by promoting export of the NFATc transcription factors out of the nucleus. *DYRK1A* is located in the Down Syndrome (DS) critical region, and elevated activity of the NFAT signaling pathway has been suggested to contribute to the pathophysiology of DS (Arron et al., 2006). Mutations in *DYRK1A* are also associated with various neurodevelopmental disorders, including ASD, ID and epilepsy (Courcet et al., 2012; J. Ji et al., 2015; Møller et al., 2008; O'Roak et al., 2012; Satterstrom et al., 2020; van Bon et al., 2016). In our collaborative study, we sought to understand the cell type-specific roles of DYRK1A during cortical development. To this end, we generated mice bearing conditional deletion of *Dyrk1a* in the lineages arising from dorsal forebrain RGCs by crossing mice with floxed *Dyrk1a* alleles to mice expressing *Cre* recombinase under the control of the endogenous *Emx1* locus (*Emx1*^{Cre}, same strategy used for conditional deletion of the *Orais* in Chapter 3). Deletion of *Dyrk1a* resulted in severe microcephaly and alterations in the cortical cytoarchitecture, including dramatic changes in the abundance of different neuronal subtypes. My contributions to this project revealed that *Dyrk1a* loss of function resulted in cell-type specific deregulation of calcium signaling in NSPCs and neurons. Specifically, we found that *Dyrk1a* loss of function resulted in severe deficits in depolarization induced calcium influx in both mouse and human NSPCs stimulated with 67mK KCl. Neurons lacking *Dyrk1a* displayed elevated intracellular calcium rises in response to depolarization, however, suggesting cell type-specific alterations in calcium signaling upon deletion of *Dyrk1a*. Indeed, our single cell sequencing analysis further revealed that these population-specific changes in calcium influx were accompanied by cell type-specific expression changes of transcripts encoding ion channels, pumps and calcium signaling molecules.

Elevated neuronal calcium rises were accompanied by significant death of neurons in mutants lacking both copies of *Dyrk1a*, both *in vitro* and *in vivo* in the cortical plate. As different patterns of calcium dynamics and activity can regulate apoptosis (Ghosh et al., 1994; N. Heck et al., 2008; Qi et al., 2020; Voigt et al., 1997; Wong Fong Sang et al., 2021), it is possible that these alterations in neuronal calcium signaling could contribute to the observed neuronal apoptosis in homozygous *Dyrk1a* conditional mutants. Finally, consistent with studies in different model organisms describing roles for *Dyrk1a* in regulating proliferation (J.-Y. Chen et al., 2013; Najas et al., 2015; Shaikh et al., 2016; Willsey et al., 2021), my contributions to this project also demonstrated altered cell cycle progression in NSPCs lacking *Dyrk1a*. Together, our results suggest that DYRK1A differentially regulates calcium dynamics in NSPCs and postmitotic neurons and plays cell type-specific roles to alter the course of cortical development.

4.2.1 Future directions: What are the consequences of DYRK1A inhibition in the developing human cortex?

DYRK1A haploinsufficiency in human patients leads to microcephaly and ID (Courcet et al., 2012; J. Ji et al., 2015; Møller et al., 2008; O'Roak et al., 2012; Satterstrom et al., 2020; van Bon et al., 2016), and studies using human induced pluripotent stem cells (iPSC) suggest impairments in neuronal differentiation in cells lacking *DYRK1A* (Cederquist et al., 2020; Willsey et al., 2021). Mechanistically dissecting how *DYRK1A* haploinsufficiency influences human cortical NSPC proliferation and differentiation is an important next step for better understanding the neurodevelopmental events that go awry in human patients with *DYRK1A* mutations. To interrogate how *DYRK1A* inhibition alters human cortical development, we have begun using primary human cortical specimens (de-identified tissue provided by the Kriegstein lab at UCSF)

treated with pharmacological inhibitors of DYRK1A (**Figure 4.2.1**) or electroporated with shRNA constructs directed against *DYRK1A* (**Figures 4.2.2-4.2.3**). Our preliminary pharmacological results on a single GW20 human cortical specimen reveals that DYRK1A inhibition using 5μM Harmine decreases the proportion of cells in G0/G1, while increasing the percentage of cells in the G2/M- and S-phases of the cell cycle. This data is consistent with the cell cycle analysis I performed on cortical cells from *Emx1*^{Cre}*;Dyrk1a*^{fl} mice for our manuscript, which further supports a role for DYRK1A in the regulation of cortical NSPC proliferation.

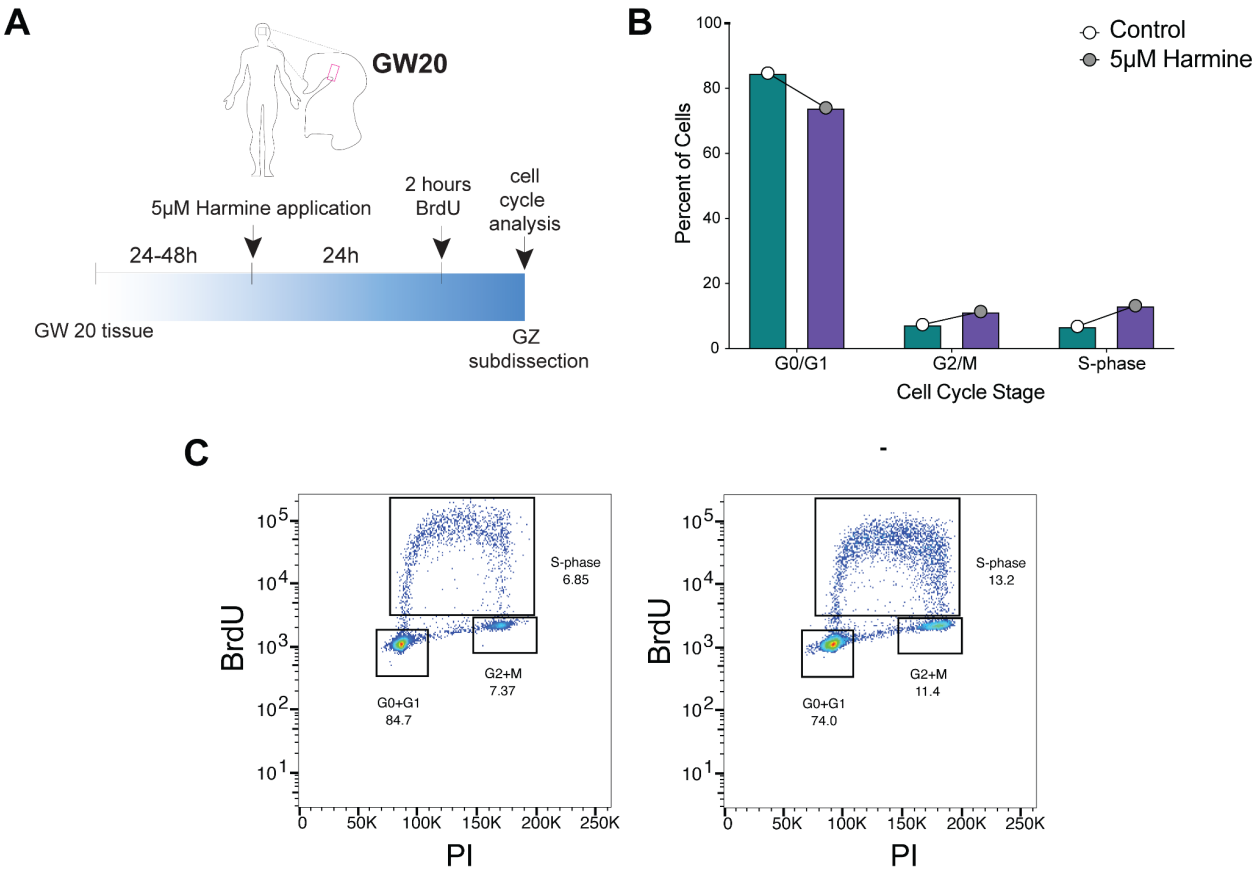

Figure 4.2.1 – DYRK1A inhibition in primary human slice cultures upregulates proliferation

(A) Schematic illustrates timeline for a single preliminary experiment. A primary GW20 human tissue specimen was vibratome-sectioned and grown as slice cultures for 24-48 hours. 5μM of the DYRK1A inhibitor Harmine was applied to the slices for 24 hours. Two hours prior to fixation for cell cycle analysis, slices were pulsed with BrdU. Germinal zones were subsequently sub-dissected and processed for cell cycle analysis.

(B, C) Flow cytometry-based DNA content analysis reveals a reduction in the percent of cells in G0/G1 and an increase in the proportion of cells in the S- and G2/M-phases upon inhibition of DYRK1A in one cortical specimen.

We also developed shRNA constructs that specifically knockdown human *DYRK1A* without altering *DYRK1B* levels (**Figure 4.2.2**). To begin to mechanistically investigate the effects of *DYRK1A* down-regulation on human NSPC proliferation, we have been optimizing methods for electroporating shRNA constructs into cells of the developing human ventricular zone (**Figure 4.2.3**). To this end, I introduced our shRNA constructs into the ventricular surface of primary human cortical specimens, using a finely pulled and beveled glass pipette. I then electroporated the shRNAs into VZ cells by placing the negatively charged electrode at the ventricular surface and the positively charged electrode closer to the pial surface. After electroporation, I cut slices from these primary pieces of tissue using a vibratome and subsequently grew them as slice cultures. Moving forward, coupling this technique or the use of CRISPR/Cas9 systems (to inactivate *DYRK1A* or introduce disease-relevant mutations) with live imaging of cells within the tissue slices will enable us to determine how loss of *DYRK1A* alters NSPC behaviors, including like proliferation, migration, and differentiation. Furthermore, immunofluorescence staining of electroporated slices grown in culture can allow us to further systematically dissect the proliferative deficits resulting from *DYRK1A* loss of function in the developing human cortex.

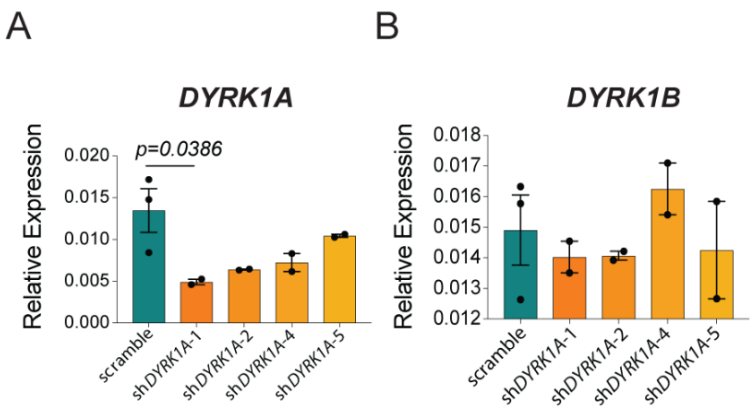

Figure 4.2.2 – shRNA constructs effectively knock down *DYRK1A* levels without affecting expression of *DYRK1B*.

A, B) shRNA constructs directed towards *DYRK1A* were cloned and transfected into SH-SY5Y human neuroblastoma cells. SH-SY5Y cells expressing shRNA constructs were then FACS isolated, and the efficiency of *DYRK1A* (**A**) and *DYRK1B* (**B**) transcript knockdown was tested using qRT-PCR. *shDYRK1A-1* was used for subsequent experiments, as it was the most effective at knocking down *DYRK1A* levels (data presented as mean ± SEM.; one-way ANOVA, Dunnett's multiple comparison test).

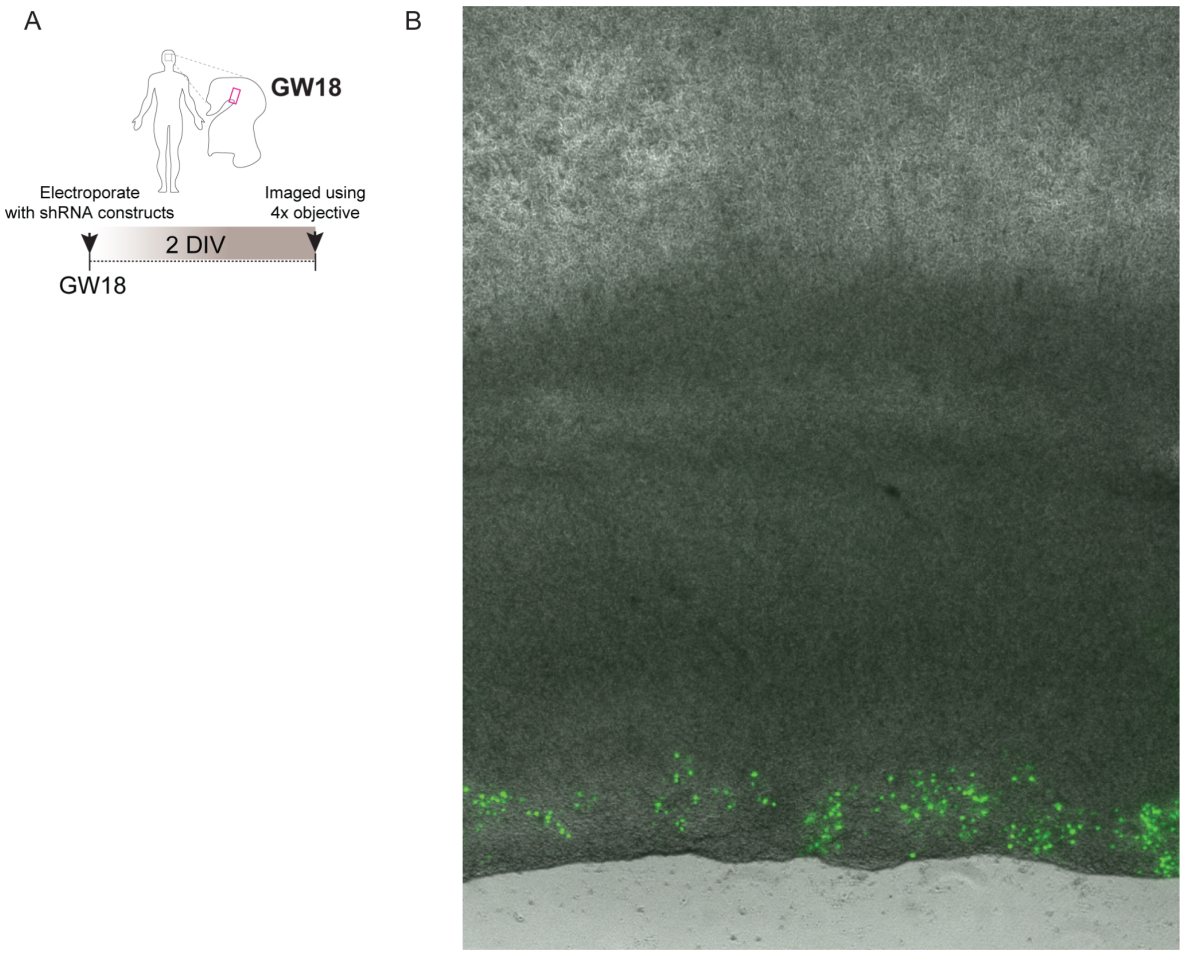

Figure 4.2.3 – Electroporation of knockdown constructs into human cortical tissue.
(A) Schematic illustrating the experimental timeline for electroporation of primary human tissue. GW18 tissue is electroporated, sectioned on a vibratome and grown as slice cultures for 2 days *in vitro* prior to imaging.
(B) Image of a vibratome-sectioned 300µm slice of GW18 human cortex electroporated with a control shRNA construct. Scramble-shRNA expressing cells can be visualized in green. The VZ, SVZ and oSVZ can be seen in this brightfield image.

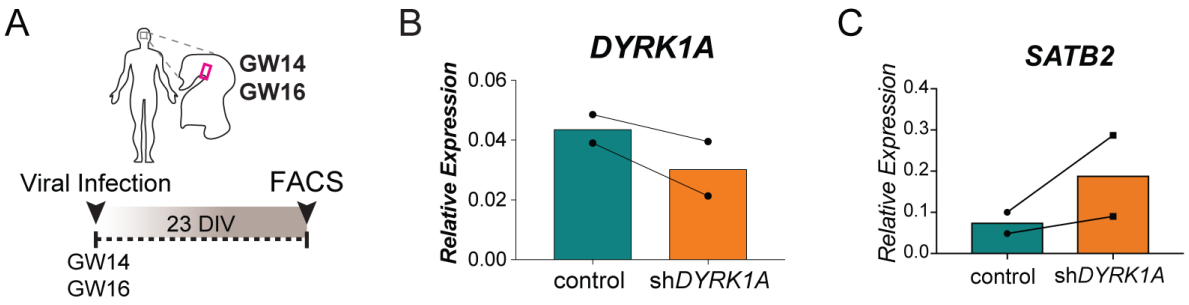

Figure 4.2.4 – Partial knockdown of *DYRK1A* increases *SATB2* transcript levels
(A) Schematic illustrating experimental timeline. Briefly, germinal zones of primary human cortical samples were dissected, dissociated and transduced with a lentivirus expressing an shRNA directed toward *DYRK1A*. Cells were grown in the absence of growth factors for 23 days, and shRNA-expressing cells were subsequently isolated for RNA isolation and qRT-PCR via FACS.
(B) *SATB2* expression levels as determined by qRT-PCR in control human cortical neurons and neurons in which *DYRK1A* expression was knocked down using shRNA..

We also have begun to use these shRNA constructs to understand how loss of *DYRK1A* influences human neuronal differentiation. To this end, I dissociated VZ/SVZ cells from primary human GW14 and 16 cortical slices, transduced them with lentivirus expressing an shRNA targeted to *DYRK1A*, and differentiated these cells *in vitro* for 23 days in the absence of growth factors. qRT-PCR analysis of FACS enriched control neurons and *DYRK1A* knockdown populations revealed a decrease in *DYRK1A* levels and an increase in *SATB2* expression, pointing to a possible role for *DYRK1A* in human neuronal differentiation (**Figure 4.2.3**). This result is reminiscent of our mutant $Emx1^{Cre};Dyrk1a^{fl}$ mice, which appear to have an increased abundance of upper layer neuronal populations and displayed elevated *Satb2* levels by both qRT-PCR and single cell RNA sequencing at P0. It is also consistent with studies using human stem cells that suggest neuronal differentiation deficits in cells lacking *DYRK1A (Cederquist et al., 2020; Willsey et al., 2021).* This preliminary observation has prompted our interest in interrogating the consequences of *DYRK1A* loss of function on the generation of deeper and upper layer neurons in the developing human cortex in future studies. To do this, as well as to interrogate the consequences of loss of *DYRK1A* on later neurodevelopmental processes like differentiation, synaptogenesis and circuit formation, we can express our shRNA constructs in a human cerebral organoid or assembloid system, which can be matured extensively in culture and coupled with electrophysiological, morphological, biochemical and immunofluorescence analyses to understand changes in later developmental processes. In complementary studies, we can examine

patient-specific iPSC-derived organoids to model cellular phenotypes associated with specific *DYRK1A* mutations.

4.2.2 What are the mechanisms underlying cell type-specific calcium signaling deficits resulting from inactivation of Dyrk1a?

We have found widespread deregulation of intracellular calcium homeostasis in the NSPCs and embryonic neurons of *Emx1-cre; Dyrk1a* conditional mutants. In the calcium imaging experiments included in our manuscript, we show that *Emx1^{Cre};Dyrk1a$^{fl/fl}$* homozygous mutant NSPCs, upon depolarization with KCl, have reduced amplitude intracellular calcium elevations in comparison to control mice. Interestingly in neurons, we show that both heterozygous and conditional mutant neurons have higher amplitude calcium rises in response to depolarizing stimuli. These changes in calcium elevations are accompanied by cell type-specific changes in ion channels and calcium signaling transcripts. Moving forward, it will be important to dissect the molecular mechanisms that result in these calcium signaling phenotypes. What molecular changes regulate the differential calcium signaling phenotypes seen in neurons and NSPCs? How do these changes in calcium affect downstream signaling mechanisms? Interestingly, our single cell RNA sequencing experiments conducted on control mice and *Emx1^{Cre};Dyrk1a^{fl}* heterozygous and homozygous mutant mice reveal that the plasma membrane calcium channels, *Orai1 and Orai3,* and the ER calcium sensor *Stim2* are significantly downregulated in RGC clusters at P0, pointing to misregulation of SOCE in proliferative cells of *Dyrk1a* mutant cortices. Further supporting a potential deficit in ER-dependent calcium signaling, we also find that *Itpr2* (encoding the IP3R Type 2) is also significantly downregulated in RGC clusters. This observation suggests the possibility of deficits in IP3-mediated calcium signaling in radial glia lacking *Dyrk1a*. Indeed,

calcium imaging experiments measuring SOCE-mediated calcium dynamics and ER calcium store content in *Emx1-cre; Dyrk1a* conditional mutant mice and controls will be imperative to develop a more complete picture of the deficits in calcium handling upon *Dyrk1a* inactivation.

It will be especially valuable to interrogate depolarization-induced calcium signaling deficits in *Dyrk1a* mutants using experiments that more closely mimic the embryonic cortical environment *in vivo*. While stimulation of neuronal cultures using potassium chloride has been widely used in the literature (Ghosh et al., 1994; Greenberg et al., 1985; Greer & Greenberg, 2008), using an endogenously-active depolarizing stimulus may give us a more physiologically accurate picture of how NSPCs and neurons lacking *Dyrk1a* respond *in vivo*. To this end, we have started investigating the effects of *Dyrk1a* deletion on neuronal calcium influx using application of GABA, an endogenous neurotransmitter that depolarizes embryonic neurons (Ben-Ari et al., 2007; Owens et al., 1996, 1999). I performed a series of Fluo-4 calcium imaging experiments on primary neuronal cultures differentiated *in vitro* for 6 days without growth factors from control, heterozygous and mutant *Emx1-Dyrk1a* NSPCs following stimulation with 30μM GABA (**Figure 4.4.3**). Similar to our experiments with KCl stimulation, our preliminary data indicate that heterozygous $Emx1^{Cre};Dyrk1a^{fl/+}$ neurons exhibited elevated intracellular calcium rises in response to GABA-mediated depolarization. Interestingly, conditional knockout mutant neurons exhibited slightly reduced amplitudes, unlike those stimulated with KCl (**Figure 4.2.3**). Though this GABA stimulation experiment is incomplete, it is important to note here a major caveat of these *in vitro* imaging experiments. Like neurons in the cortical plate *in vivo*, our *in vitro* neuronal cultures from *Dyrk1a* conditional knockout mice displayed substantial neuronal death. Thus calcium imaging traces from neuronal cultures derived from full conditional mutant mice are likely a reflection of the remaining surviving neurons. This phenomenon may explain differences seen in responses to

depolarization with KCl and our preliminary experiments using GABA in *Emx1*^{Cre}*;Dyrk1a*$^{fl/fl}$ full conditional mutant mice. Nevertheless, these results support that *Dyrk1a* loss of function leads to deregulation of neuronal calcium signaling mechanisms.

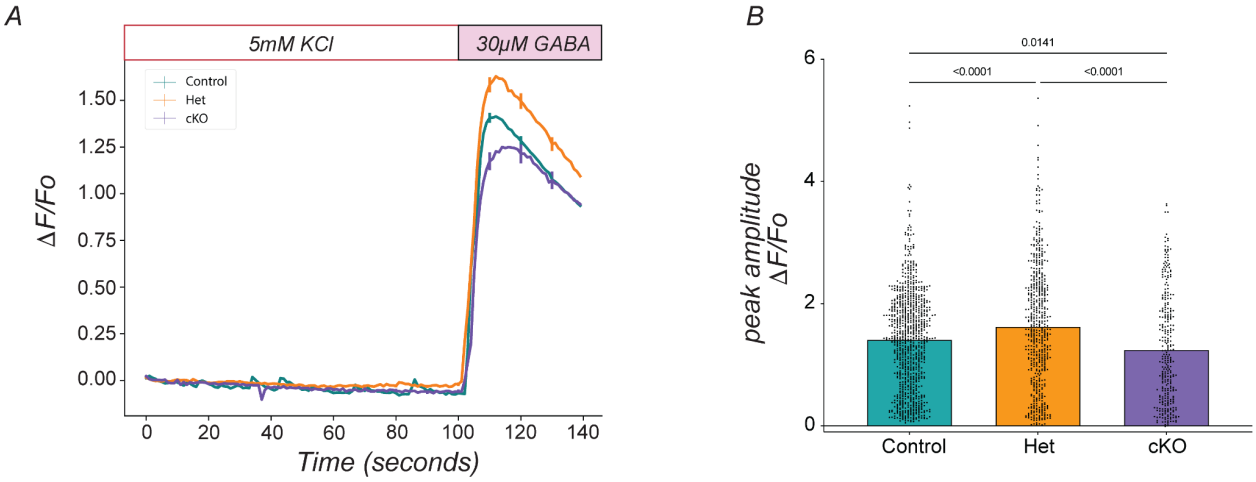

Figure 4.2.5 – Heterozygous *Emx1*^{Cre}*;Dyrk1a*$^{fl/+}$ cortical neurons exhibit elevated intracellular calcium rises in response to GABA-mediated depolarization
(A) Mean calcium response traces are shown for control (Ctrl, *teal*, *Dyrk1a*$^{fl/+}$ or *Dyrk1a*$^{fl/fl}$), heterozygous (Het, *orange*, *Emx1*^{Cre}*;Dyrk1a*$^{fl/+}$), and cKO (*violet*, *Emx1*^{Cre}*;Dyrk1a*$^{fl/fl}$) neurons (data presented as mean ± SEM; Ctrl: 899 cells, 2 mice; Het: 554 cells, 2 mice; cKO: 306 cells, 3 mice).
(B) Heterozygous *Dyrk1a mutant* neurons display significantly elevated peak amplitudes compared to control neurons after GABA depolarization. (data presented as mean ± SEM; one way ANOVA, post-hoc Tukey's multiple comparisons test).

4.3 – Repurposing of calcium-regulated developmental mechanisms in adult disease states?

Deregulation of calcium signaling is implicated in adult disease states. We are thus also actively investigating disease contexts in which developmental mechanisms may be reactivated to

contribute to disease progression. Specifically, we hypothesize that developmental calcium signaling mechanisms that regulate cell cycle progression may be hijacked to influence abnormal proliferation in disease states like glioma. Recent studies have revealed that transcriptomic, morphological and behavioral similarities exist between stem-like populations in the developing brain and cancer stem-like cells in primary glioma samples (Wang et al., 2020; Bhaduri et al., 2020). For example, cells resembling embryonic neural stem cells have been identified in primary glioma specimens and are thought to contribute to glioma development and maintenance. Live imaging studies have shown that these glioma stem cells display features of RG- and oRG-like function, including migratory behaviors like interkinetic nuclear migration and mitotic somal translocation (Wang et al., 2020; Bhaduri et al., 2020). Similarities to ventricular radial glia can also be seen in the calcium signaling properties of these cells, as they display spontaneous synchronous calcium transients that propagate across gap junction-coupled glioma cells. Given these similarities, we have begun investigating whether the calcium-sensitive components that we are learning play important roles during development are also utilized by proliferative glioma cells.

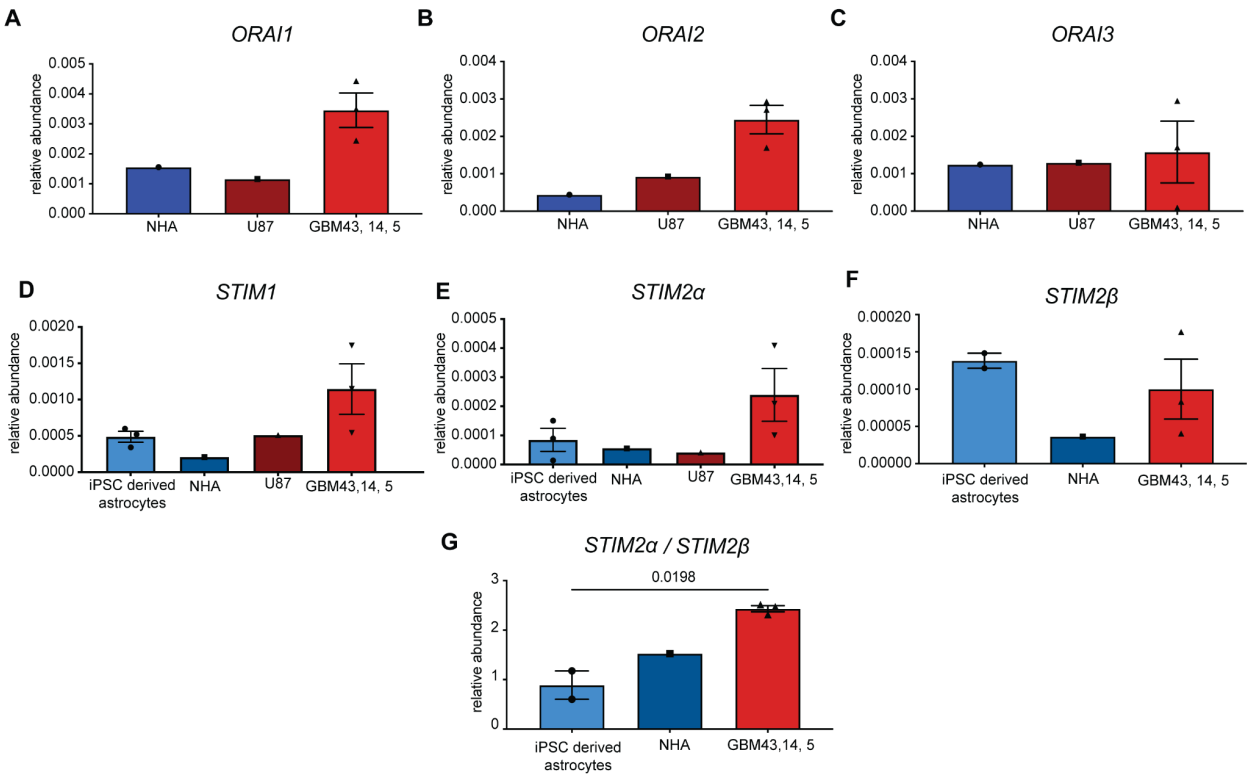

Figure 4.3.1 – SOCE mediators are upregulated in primary human glioma cell lines
A-G) qRT-PCR for regulators of SOCE, including *ORAI1-3* **(A-C)**, *STIM1* **(D)**, *STIM2*α **I**, and *STIM2*β **(F)** in primary glioblastoma-derived cell lines (data presented as mean ± SEM, one way Anova, post-hoc Tukey's multiple comparisons test).

To begin to understand roles for SOCE in glioma biology, we examined the expression of genes that encode SOCE mediators in a normal human astrocyte (NHA) cell line, hIPSC-derived astrocytes, a glioblastoma (GBM) cell line (U87), and cell lines derived from three primary GBM samples. De-identified glioma cell lines were provided by the Lim lab at UCSF, and de-identified human IPSC-derived astrocytes were provided by Ilyas Singec at the NCATS/NIH. We found elevated expression levels of *STIM1*, *ORAI1* and *ORAI2,* positive regulators of SOCE, in primary GBM derived cell lines (**Figure 4.3.1**). Intriguingly, we observed that the ratio of *STIM2α*, the *STIM2* splice variant known to activate SOCE, to *STIM2β*, the variant that inhibits SOCE, is increased in primary glioma cell lines. This compelling preliminary data suggests that calcium signaling mechanisms regulating normal proliferation in the developing brain and alternative

splicing of calcium-related transcripts may be deregulated or reactivated in glioma cells to favor SOCE activators and promote excess proliferation. We are especially interested in following up these initial observations to interrogate the expression of other calcium-permeant ion channels and calcium sensitive-transcription factors in glioma specimens. Indeed, this will require investigating the full splice isoform diversity of different calcium signaling molecules in both normal and disease contexts.

Insights into the regulation of calcium signaling in the embryonic cortex are essential to understand the fundamental mechanisms driving development, but may also be important in developing therapeutic strategies to target developmental mechanisms that are hijacked in disease states. Understanding the functional consequences of the expression of these calcium signaling molecules and their splice variants will enable us to develop a more comprehensive model of how calcium signaling molecules work in concert with each other to influence cellular behaviors during development and in disease.

Appendix A – Publications

A.1 Journal Articles

1. Petrova R, **Arjun McKinney A**, Wu B, Torres T, Hamid S, Delgado RN, Ki C, Su Z, Qui L, Pippin H, Nowakowski TJ, Ellegood J, Lim DA, Graef I, Darmanis S & Panagiotakos G. "DYRK1A Kinase Regulates Cortical Development via NFAT-mediated Cell-specific Modulation of Calcium Signaling." Manuscript being finalized for submission.

   *This work is not included in the thesis, but a discussion of some of the key findings that I contributed to, as well as complementary experiments that I spearheaded, is included in the final chapter. For this study, I performed and analyzed all calcium imaging experiments in control and *Dyrk1a* mutant NSPCs and neurons, which revealed cell type-specific changes in depolarization-dependent calcium elevations upon deletion of *Dyrk1a*. I performed, together with Ralitsa Petrova, the cell isolation and sample preparation for single cell RNA sequencing of control and mutant mice, and Bing Wu and I contributed to all single cell sequencing data analysis. I also performed all experiments conducted in human primary tissue, including calcium imaging experiments in human NSPCs, and I generated human lentiviral shRNA constructs to test how inhibition of *DYRK1A* alters human cortical development. Finally, I performed flow cytometry based cell cycle analysis which revealed that conditional deletion of *Dyrk1a* results in an increased fraction of proliferative cells.

2. Andrews MG, Siebert C, Wang L, White M, Ross J, Morales R, Donnay M, Bamfonga G, Mukhtar T, **Arjun McKinney A**, Gemenes K, Wang S, Bi Q, Crouch EE, Parikshak N,

Panagiotakos G, Huang E, Bhaduri A, Kriegstein AR "LIF signaling regulates outer radial glial to interneuron fate during human cortical development." Manuscript in submission.

* This work is not included in this thesis.

3. **Arjun McKinney A,** Petrova, R., & Panagiotakos, G. "Calcium and activity-dependent signaling in the developing cerebral cortex." Development, *149*(17). September 2022.

*This work is included as Chapters 1 and 2 of this thesis.

4. Panagiotakos G, Haveles C, **Arjun A**, Petrova R, Rana A, Portmann T, Paşca SP, Palmer TD, Dolmetsch RE. "Aberrant calcium channel splicing drives defects in cortical differentiation in Timothy syndrome." Elife. 8:e51037. December 2019.

* This work is not included in this thesis.

5. Mayer S, Chen J, Velmeshev D, Mayer A, Eze UC, Bhaduri A, Cunha CE, Jung D, **Arjun A**, Li E, Alvarado B, Wang S, Lovegren N, Gonzales ML, Szpankowski L, Leyrat A, West JAA, Panagiotakos G, Alvarez-Buylla A, Paredes MF, Nowakowski TJ, Pollen AA, Kriegstein AR "Multimodal Single-Cell Analysis Reveals Physiological Maturation in the Developing Human Neocortex." Neuron. 102(1):143-158.e7. April 2019.

* This work is not included in this thesis. I performed calcium imaging in cells from the mouse embryonic cortex and created a preliminary version of an immunofluorescence/calcium imaging pipeline that allowed co-registration of the calcium imaging field of view to its corresponding immunofluorescence image. My calcium

imaging results helped support the conclusion that HTR2A signaling may be specific to primates.

A.2 Conference posters, talks and abstracts

1. **Arjun A*,** Tong J, Launer S, Pedrozo V, Petrova R, Khan Y, and Panagiotakos G. "Investigating the roles of Store-Operated Calcium Entry during development of the human and mouse cerebral cortex" International Society for Stem Cell Research Conference, Virtual Conference, 2021
2. **Arjun A***, Panagiotakos G "Examining the role of Store-Operated Calcium Entry during embryonic corticogenesis" Developmental and Stem Cell Biology Retreat, Santa Cruz, CA, 2019
3. **Arjun A*,** Launer S, Tong J, Petrova R, Khan Y, Rana A, Dua P, and Panagiotakos G. "Dissecting the regulation and function of Store-Operated Calcium Entry during cortical development" Society for Developmental Biology, Boston, MA, 2019
4. **Arjun A*,** Launer S, Tong J, Petrova R, Dua P, Khan Y, and Panagiotakos G. "Dissecting the roles of Store-Operated Calcium Entry during the development of the mammalian cerebral cortex" International Society for Stem Cell Research Conference, Los Angeles, CA, 2019
5. **Arjun A***, Panagiotakos G "Examining the role of Store-Operated Calcium Entry during embryoic corticogenesis" Parnassus Research in Progress Seminar, UCSF, 2019
6. Petrova R, **Arjun A***, Wu B, Torres T, Su Z, Ki C, Pippin H, Graef I, Darmanis S, Panagiotakos G. "Defining the Role of L-Type Calcium Channels and Calcineurin/NFAT Signaling in Neuronal Specification" West Coast Society for Developmental Biology, Cambria, CA, 2019

7. **Arjun A*,** Launer S, Tong J, Petrova R, Khan Y, and Panagiotakos G "Dissecting the Roles of Store-Operated Calcium Entry during Development of the Cerebral Cortex" Society for Neuroscience, San Diego, CA, 2018
8. **Arjun A***, Panagiotakos G "Regulation of embryonic cortical development by store operated calcium entry" Stem Cell Workshop, San Francisco, CA, 2018
9. **Arjun A***, Panagiotakos G "Examining the role of the ER calcium sensor Stim2 during embryonic neurogenesis" Stem Cell Workshop, San Francisco, CA, 2017

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