Calcium Signaling in Neocortical Development

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ABSTRACT: **The calcium ion (Ca^{2+}) is an essential second messenger that plays a pivotal role in neurogenesis. In the ventricular zone (VZ) of the neocortex, neural stem cells linger to produce progenitor cells and subsequently neurons and glial cells, which together build up the entire adult brain. The radial glial cells, with their characteristic radial fibers that stretch from the inner ventricular wall to the outer cortex, are known to be the neural stem cells of the neocortex. Migrating neurons use these radial fibers to climb from the proliferative VZ in the inner part of the brain to the outer layers of the cortex, where differentiation processes continue. To establish the complex structures that constitute the adult cerebral cortex, proliferation, migration, and differentiation must be tightly controlled by various signaling events, including cytosolic Ca^{2+} signaling. During development, cells regularly exhibit spontaneous Ca^{2+} activity that stimulates downstream effectors, which can elicit these fundamental cell processes. Spontaneous Ca^{2+} activity during early neocortical development depends heavily on gap junctions and voltage dependent Ca^{2+} channels, whereas later in development neurotransmitters and synapses exert an influence. Here, we provide an overview of the literature on Ca^{2+} signaling and its impact on cell proliferation, migration, and differentiation in the neocortex. We point out important historical studies and review recent progress in determining the role of Ca^{2+} signaling in neocortical development.**
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INTRODUCTION

The first report of the calcium ion (Ca^{2+}) as a signaling mediator in cells was made more than one-hundred years ago, in 1883, when Sidney Ringer discovered that hearts contracted in London tap water, but failed to do so in distilled water (Ringer, 1883; Carafoli, 2003). It was not until 1959 that Nishi and coworkers demonstrated for the first time Ca^{2+} currents in neurons (Koketsu et al., 1959a, b). Using spinal ganglion cells isolated from frogs they showed that action potentials were eliminated when Ca^{2+} was withdrawn from the sodium-free solution. One year before Nishi's reports of Ca^{2+} currents in

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neurons, in 1958, Fatt and Ginsborg showed for the first time the existence of Ca^{2+}-mediated action potentials in crayfish muscles (Fatt and Ginsborg, 1958). Almost two decades later, in 1976, another fundamental study by Kuba and Nishi suggested that Ca^{2+} could trigger membrane potential oscillations in neurons (Kuba and Nishi, 1976). These very important studies were instrumental for the field of Ca^{2+} signaling in neural cells. Another study that had a major impact on the field of Ca^{2+} signaling was Roger Tsien's development of new Ca^{2+} indicators based on 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid in 1980, which could be used to monitor real-time fluctuations of Ca^{2+}-ions in individual living cells (Tsien, 1980). In 1991, using Tsien's invention Fura-2, Yuste and Katz detected neurotransmitter-evoked Ca^{2+} activity in the postnatal (P1–P7) developing neocortex of rats (Yuste and Katz, 1991). A few years later Spitzer and coworkers demonstrated that Ca^{2+} signaling could affect neuronal development, when they showed that neurotransmitter respecification in spinal neurons was regulated by the frequency of spontaneous Ca^{2+} transients (Gu et al., 1994; Gu and Spitzer, 1995).

Today, we know that Ca^{2+} is a highly versatile intracellular signaling messenger that operates over a wide temporal range to regulate many different cellular processes during the entire lifespan of the cell (Berridge et al., 1998). The Ca^{2+} ion is ideally suited for signal transduction as the cytosolic resting concentration is low ($\sim10^{-7}$ *M*) and the extracellular concentration is high ($\sim10^{-3}$ *M*); therefore, Ca^{2+} fluctuations can be generated and detected with only a relatively small increment of Ca^{2+} in the cytoplasm. Cells have extensive Ca^{2+}-signaling toolkits to assemble signaling systems with very different spatial and temporal dynamics (Berridge et al., 2003; Clapham, 2007). Under certain circumstances, the cytosolic Ca^{2+} concentration can start to oscillate and activate specific cellular programs, depending on the frequency of the signal (Uhlen and Fritz, 2010). Transcription factors, for example, nuclear factor of activated T-cells, can decode such oscillatory Ca^{2+} signals (Uhlen et al., 2006; Smedler and Uhlen, 2014). Recently, the fate-determination transcription factor Satb1 (special AT-rich binding protein) was shown to be activity-modulated for the terminal differentiation and connectivity of medial ganglionic eminence-derived cortical interneurons (Close et al., 2012). Interestingly, several other fate-determination transcription factors were recently reported to exhibit an oscillatory expression pattern in neural progenitor cells (Imayoshi et al., 2013). Using optogenetics to control expression of Ascl1 (achaete-scute family bHLH transcription factor 1), Kageyama and coworkers found that an oscillatory expression maintained proliferating neural progenitor cells, whereas a sustained expression stimulated neuronal fate determination.

During early neocortical development, neuroepithelial cells transform into neural progenitor cells, termed radial glial cells, which play a major role at multiple steps of brain development (Gotz and Huttner, 2005). Radial glial cells were first mentioned by Pasko Rakic in 1971 as glial cells with cell bodies situated near the ventricle and long fibers that extend to the pial surface, providing scaffolds for migrating neurons (Rakic, 1971; Rakic, 1972; Levitt and Rakic, 1980). In recent years, using advanced molecular tools and time-lapse imaging techniques, we have learned that radial glial cells are actually neural progenitors that can divide and produce migrating neurons (Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001). Radial glial cells located in the proliferative zone at the innermost (apical) side of the developing cortex, termed the ventricular zone (VZ), produce virtually all glutamatergic neurons that form the basic layer structure of the cortex. The VZ is a pseudostratified epithelium because radial glial nuclei are positioned at different levels as a result of interkinetic nuclear migration (Sauer, 1935; Taverna and Huttner, 2010). Herein, we will discuss the influence of Ca^{2+} signals on cell proliferation, migration, and differentiation during neocortical development.

SPONTANEOUS CALCIUM ACTIVITY

The Ca^{2+} second messenger system constitutes a highly versatile signal transduction pathway (Berridge et al., 2000). Although the Ca^{2+} concentration is strongly regulated [as an excessively high Ca^{2+} concentration is toxic to the cell (Orrenius et al., 2003)], intracellular Ca^{2+} levels may occasionally start to vary in cycles in both time and space; forming what is known as Ca^{2+} waves and Ca^{2+} oscillations (Uhlen and Fritz, 2010). When examining cytoplasmic Ca^{2+} levels in single cells under the microscope, spontaneous Ca^{2+} oscillations are frequently observed; that is, without the experimenter influencing the system under investigation. Spontaneous Ca^{2+} oscillations occur extensively during the development of the nervous system, where it regulates fundamental cell processes, including proliferation, migration, and differentiation (Blankenship and Feller, 2010; Rosenberg and Spitzer, 2011; Yamamoto and Lopez-Bendito, 2012). Intriguingly, this

activity-driven control of development is complementary to the genetic program (Spitzer, 2006).

Neurons are special with regard to Ca^{2+} signaling, as the Ca^{2+} concentration is not only affected by intracellular stores, but also by the plasma membrane potential (Berridge, 1998). Thus, Ca^{2+} can be considered an intermediate currency between electric signaling and physiological responses such as transcription and exocytosis. In network circuits of immature neurons in the retina (Meister et al., 1991), spinal cord (Gonzalez-Islas and Wenner, 2006), auditory nerve (Tritsch et al., 2007), hippocampus (Garaschuk et al., 1998), and cerebellum (Watt et al., 2009), spontaneous electrical activity (and consequently spontaneous Ca^{2+} activity) has been discovered and characterized. In principal, spontaneous network activity could arise as a result of two different mechanisms: either certain pacemaker cells drive the activity of the entire network, or the properties of the network itself, as an intrinsic property, lead to spontaneous activity. Indeed, in the heart, differentiated pacemaker cells control the electrical activity of the entire organ (Mangoni et al., 2006), whereas in the inferior olivary nucleus of the medulla oblongata, spontaneous activity arise as a result of network properties (Manor et al., 1997).

In general, individual cells are highly competent at regulating their own Ca^{2+} level. However, in an organism, cells are interconnected and exposed to an extracellular environment. Under these circumstances, Ca^{2+} fluctuations can occur as a consequence of events outside of cell-autonomous control. For instance, minute instabilities in the cytosolic Ca^{2+} concentration may be amplified by Ca^{2+}-induced Ca^{2+} release, especially in immature networks. One of the first reports of spontaneous Ca^{2+} activity in the developing cerebral cortex was by Rafael Yuste et al. in the laboratory of Lawrence C. Katz in 1992 (Yuste et al., 1992). They showed that neuronal domains, prototypes for future columnar units, are established during development *via* neurotransmitter-modulated spontaneous Ca^{2+} activity.

The literature reviewed in this article suggests that the driving mechanism of spontaneous Ca^{2+} activity in neocortical development varies at the different developmental phases: during the early proliferative/migratory phase the activity is driven by gap-junctions and voltage dependent Ca^{2+} channels, whereas later in development, during the migratory/differentiation phase, local neurotransmitters, and synapses modulate the spontaneous Ca^{2+} activity (Fig. 1). Interestingly, two distinct systems for spontaneous activity have been demonstrated in the developing mouse cortex when gamma-aminobutyric acid

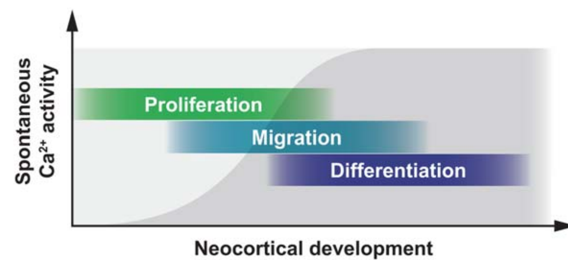

Figure 1 Spontaneous Ca^{2+} activity during neocortical development. Neocortical development involves sequential but overlapping phases of neuronal proliferation, migration, and differentiation. Spontaneous Ca^{2+} activity during early neocortical development depends heavily on gap junctions and voltage dependent Ca^{2+} channels (light gray area), whereas later in development neurotransmitters and synapses exert an influence (dark gray area).

(GABA)-ergic neurotransmission was genetically eliminated (Easton et al., 2014).

CALCIUM SIGNALING AND NEURAL PROLIFERATION

One of the first observations of Ca^{2+} activity in the developing cerebral neocortex was performed in Kriegstein's laboratory in 1995 (LoTurco et al., 1995). Using GABA and glutamate, these authors detected increases in intracellular Ca^{2+} in VZ cells of E17 embryos, in part through activation of voltage-gated Ca^{2+} channels, and suggested that these signaling systems are involved in proliferation. Later, the same group reported synchronous spontaneous Ca^{2+} increases in pairs of adjacent cells of the neocortical VZ (Owens and Kriegstein, 1998). These synchronized cell pairs were identified as dividing M-phase cells, located at the apical surface of the VZ. In another important study published in 2004, Kriegstein's laboratory confirmed that the spontaneously occurring Ca^{2+} waves in the VZ were correlated with proliferation and that hemichannels were crucial for this signaling event (Weissman et al., 2004). Whereas hemichannels in the plasma membrane of two neighboring cells may form a gap junction, a single hemichannel may release intracellular molecules into the intercellular space. Gap junctions are multifaceted regulators of embryonic cortical development (Elias and Kriegstein, 2008).

Adenosine triphosphate (ATP) signaling has been shown to be involved in proliferation, as ATP is released through hemichannels it activates purinerigc receptors resulting in inositol 1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} increases (Scemes et al.,

2003; Weissman et al., 2004; Pearson et al., 2005). Furthermore, purinergic signaling drives the expansion of VZ neural stem and progenitor cells (Lin et al., 2007). Neural progenitors express purinergic P2Y receptors and mobilize intracellular Ca^{2+} in response to ATP. Moreover, the pituitary adenylate cyclase-activating peptide ligand/type 1 receptor signaling system is reported to play a key role in controlling the proliferation of the cortical astrocyte lineage precursors via Phospholipase C (PLC)/$InsP_3$-dependent Ca^{2+} signaling pathways (Nishimoto et al., 2007). In human fetal forebrain-derived neural progenitors, Ca^{2+} signaling lengthens the cell cycle and enhances the generation of neurons (Garcia-Garcia et al., 2012). This increase in the number of neurons is a result of more intermediate progenitor proliferation. In another study, inhibition of N-methyl-D-aspartic acid (NMDA) receptors induced delayed neuronal maturation and sustained proliferation of progenitor cells in neocortical development (Hirasawa et al., 2003).

Embryonic stem cell-derived neural progenitors were found to form networks exhibiting synchronous Ca^{2+} activity that stimulated cell proliferation (Malmersjo et al., 2013). The Ca^{2+} activity was dependent on gap junctions (connexin 43), contrary to studies mentioned above this signaling was independent of both release of ATP through hemichannels and intracellular Ca^{2+} stores. Instead a depolarizing current was spread from cell to cell via gap junctions leading to activation of voltage-dependent Ca^{2+} channels in the plasma membrane. Furthermore, inhibition of these signal events abolished the Ca^{2+} activity, suppressed proliferation, and perturbed embryonic cortical layer formation. Performing cross-correlation analysis on these Ca^{2+} signaling networks revealed highly correlated activities in small-world networks that follow a scale-free topology (Smedler et al., 2014). Interestingly, graph theory states that such network designs are highly effective for biological systems, as these networks are more robust to perturbations, for example, mutations or viral infections, than other network architectures (Barabasi and Oltvai, 2004).

The link between spontaneous Ca^{2+} signaling and neural proliferation has also been shown using mathematical modeling (Barrack et al., 2014). Here the authors apply bifurcation analysis, suggesting that Ca^{2+} signaling has only a minor effect on the length of the cell cycle. However, their analyses also suggest that quiescent cells (in G0 phase) are reentering the cell cycle via a Ca^{2+} signaling and cyclin D dependent mechanism, thereby enhancing proliferation.

CALCIUM SIGNALING AND NEURAL MIGRATION

One of the first reports of modulation of neuronal migration by Ca^{2+} signaling was by Komuro and Rakic in 1992 (Komuro and Rakic, 1992). In mouse cerebellar slice preparations, they observed that postmitotic granule cells initiated their migration only after they started to express N-type Ca^{2+} channels.

During cortical development, progenitor neurons form multipolar shaped cells that slowly migrate in the subventricular zone and intermediate zone, where many intermediate progenitor cells linger (Tabata and Nakajima, 2003; Noctor et al., 2004). Rakic and coworkers reported that intermediate progenitor cells, unlike the postmitotic neurons that tend to lose the ATP-mediated Ca^{2+} response, continued to express the purinergic $P2Y_1$ receptor and that inhibiting spontaneous Ca^{2+} activity retarded migration of intermediate progenitors to the subventricular zone (Liu et al., 2008). The same group used short hairpin RNA-mediated knockdown of connexin 43 to show that gap junctions/hemichannels are involved in Ca^{2+} signaling and the apical phase of the interkinetic nuclear migration in VZ progenitors (Liu et al., 2010).

Beneath the cortical plate, postmitotic neurons transform into unipolar-shaped cells and attach to the radial glial fibers to rapidly migrate along it until near the pial surface (Rakic, 1972; Nadarajah et al., 2001). Although hemichannels formed of connexin 26 and connexin 43 are shown to be necessary for radial glial-guided neuronal migration in the neocortex (Elias et al., 2007), pharmacological inhibition of the $P2Y_1$ receptor with suramin or shRNA knockdown had no affect on the migration process. These results suggest that Ca^{2+} waves mediated by connexin-hemichannels are the not the main player in neuronal migration.

There are reports that extracellular glutamate and GABA increase intracellular Ca^{2+} signaling *via* the NMDA and $GABA_A$ receptors (Owens et al., 1996; Behar et al., 1999). These results show that NMDA promotes neuronal migration (Behar et al., 1999; Hirai et al., 1999), whereas GABA hampers migrating cortical neurons (Behar et al., 2000). *In vivo* application of antagonists for the NMDA receptor (MK801) (Reiprich et al., 2005) and the $GABA_A$ receptor (bicuculline) (Heck et al., 2007) both disrupts neuronal migration in the cerebral cortex. Despite many observations, the exact role and mechanism of NMDA and GABA in neuronal migration are still controversial, partially because the transgenic mice models NMDAR-1 (Messersmith et al., 1997)

and GAD65/GAD67 double knockout mice (Ji et al., 1999) do not show migratory defects.

Ambient GABA and glutamate initially stimulate the motility of interneurons through both $GABA_A$ and AMPA/NMDA receptor activation (Bortone and Polleux, 2009). When the migrating interneurons reach the cortex, upregulation of the potassium-chloride cotransporter KCC2 decreases the frequency of spontaneous Ca^{2+} activity, which reduces the motility of these interneurons. Real-time imaging of tangentially migrating GABAergic interneuron in the neocortical marginal zone, revealed that the motility rate is significantly reduced when intracellular Ca^{2+} is chelated (Inada et al., 2011).

During embryogenesis, the receptor tyrosine kinase rearranged during transfection (RET) plays an essential role in regulating cell proliferation, differentiation, and migration (Arighi et al., 2005; Pozas and Ibanez, 2005). On glial cell-derived neurotrophic factor stimulation, RET induces Ca^{2+} signaling and regulates neocortical neuronal progenitor migration through its PLC gamma-binding domain (Lundgren et al., 2012). A recent study shows that receptor tyrosine kinase and PLC signaling are restricted to the leading edge, triggering local Ca^{2+} pulses, local depletion of Ca^{2+} in the endoplasmic reticulum, and local activation of the mediator of store-operated Ca^{2+} influx, STIM1, thus supporting pulsatile front retraction and adhesion (Tsai et al., 2014). This showed that cells use an integrated Ca^{2+} control system, consisting of polarized Ca^{2+} signaling proteins and second messengers, that synergistically promote directed cell migration.

CALCIUM SIGNALING AND NEURAL DIFFERENTIATION

In a study from 1985 on calmodulin expression in nerve growth cone preparations from fetal rat brains, Hyman and Pfenninger suggest a role for intracellular Ca^{2+} in neurite sprouting (Hyman and Pfenninger, 1985). Today, it is generally accepted that Ca^{2+} signaling is involved in dendritic development (Konur and Ghosh, 2005). In a recent study, Yoshiaki Tagawa and coworkers report that postmigratory neurons in layer II/III exhibit more vivid spontaneous Ca^{2+} activity than migrating neurons (Bando et al., in press). Increasing the neural activity of migrating neurons by overexpressing a bacterial voltage-dependent sodium channel (NachBac) hamper migration and trigger premature dendrite outgrowth before neurons arrive at layer II/III. The same group demonstrated a similar migration disability when spontaneous Ca^{2+} activity was increased by knocking down the two-pore-domain (KCNK) potassium channel KCNK9 (Bando et al., 2014). KCNK channels are critical determinants of neuronal excitability in the mature cerebral cortex, and KCNK9 (TASK3) is responsible for a maternally transmitted mental retardation syndrome (Barel et al., 2008).

A study on immature cortical neurons showed that the steroid hormone ouabain stimulates dendritic growth (Desfrere et al., 2009). Ouabain, at low doses that do not inhibit Na,K-ATPase, triggers Ca^{2+} oscillations that activate the transcription factor 3'-5'-cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and mitogen-activated protein kinases through Ca^{2+}/calmodulin-dependent protein (CaM) kinases to stimulate neural differentiation. Ghosh and coworkers showed that CaM kinase IV and CREB play a critical role in mediating Ca^{2+}-induced dendritic growth in immature cortical neurons (Redmond et al., 2002). By depolarizing cells, they enhance Ca^{2+} activity, resulting in increased dendritic growth. In another study, CaM kinase alpha and CaM kinase gamma were shown to regulate axonal and dendritic morphogenesis of cortical neurons, respectively (Ageta-Ishihara et al., 2009). Here, Ca^{2+} signaling was stimulated by muscimol, a $GABA_A$ receptor agonist. Moreover, the amplitude of spontaneous Ca^{2+} oscillations has been reported to modulate neuronal differentiation of early postnatal neocortical pyramidal cells (Hamad et al., 2014). Expressing the cytoplasmic tail of the AMPA receptor regulatory protein type I TARP γ-8 increases the amplitude but not the frequency of spontaneous Ca^{2+} transients, resulting in greater dendritogenesis of immature pyramidal neurons.

Protein ubiquitination by Nedd4 (neuronal precursor cell expressed and developmentally downregulated protein) is known to modulate neurite growth and arborization in mammalian neurons (Drinjakovic et al., 2010; Kawabe et al., 2010). Nedd4 is acting on the phosphatase and tensin homolog, a negative regulator of the PI3K pathway, and reduces the activity of the Rap2 effector kinases of the serine/threonine kinase TNIK family to promote dendrite growth. These differentiation processes are likely to be modulated by intracellular Ca^{2+} signaling, as Ca^{2+} releases the C2 domain-mediated auto-inhibition of Nedd4 (Wang et al., 2010).

FUTURE PERSPECTIVES

Recent technological advances have opened up new possibilities to both monitor and manipulate Ca^{2+}

signals in cultured cells and *in vivo*. Genetically encoded Ca^{2+} indicators consist of different combinations of fluorescent proteins fused to a Ca^{2+}-binding domain (e.g., calmodulin), where the fluorescent signal is dependent on binding of Ca^{2+} ions. Iterative improvements in the genetically encoded Ca^{2+} indicators over several years have resulted in powerful tools suitable for monitoring Ca^{2+} signaling and, hence, neural activity (Miyawaki et al., 1997; Chen et al., 2013). In particular, the recent developments of GCaMP have the sensitivity, dynamic range, and kinetics needed to accurately monitor Ca^{2+} activity in neurons (Chen et al., 2013). While Ca^{2+} indicators enable visualization of single-cell activity, optogenetics provide powerful tools to manipulate intrinsic neuronal activity using light, either in cultured cells or *in vivo* (Tye and Deisseroth, 2012). By using optogenetics in specific cell types using cell specific promoters, restricted regions of the brain can be stimulated or inhibited at particular time points.

Monitoring and manipulating ion flows is important; however, there is also a need to decipher both the upstream signal (i.e., how the Ca^{2+} signal is generated) and the downstream signal (i.e., which signaling proteins are effected by Ca^{2+}). To further explore how different proteins involved in Ca^{2+} signaling modulate neural development, specific genes can be manipulated using emerging genetic technologies, such as clustered regularly interspaced short palindromic repeats (CRISPRs) (Swiech et al., in press) or CRISPR interference (Qi et al., 2013). Finally, the recent improvements in methods that render biological samples optically transparent while preserving fluorescent signals [e.g., CLARITY (Chung et al., 2013) and SCALE (Hama et al., 2011)] could prove to be valuable when used in combination with genetic manipulations, such as CRISPRs or Optogenetics, to further our understanding of neocortical development.

CONCLUSIONS

Exploring the role of Ca^{2+} signaling in neocortical development provides a better understanding of the formation and functions of the healthy brain, and might also provide key insights into the pathogenesis of neurodegenerative and neurodevelopmental disorders. There is mounting evidence that spontaneous Ca^{2+} activity is crucial at multiple steps during cerebral cortex formation, including the proliferation, migration, and differentiation phases. These reports suggest that spontaneous Ca^{2+} activity during early neocortical development depends heavily on gap junctions and voltage dependent Ca^{2+} channels, whereas later in development neurotransmitters and synapses exert an influence. Further investigations, however, are required to fully understand the regulatory role of Ca^{2+} signaling during the many steps and processes of neocortical development. The rapid development of molecular tools and imaging techniques will surely shed light on the multifaceted Ca^{2+} ion and its influence on neocortical development.

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