HHS Public Access

Author manuscript

Neuron. Author manuscript; available in PMC 2023 January 19.

Published in final edited form as:

Neuron. 2022 January 19; 110(2): 195–208. doi:10.1016/j.neuron.2021.10.035.

A matter of space and time: emerging roles for disease-associated proteins in neural development

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Abstract

Recent genetic studies of neurodevelopmental disorders point to synaptic proteins and ion channels as key contributors to disease pathogenesis. While many of these proteins, such as the L-type calcium channel $Ca_V1.2$ or the postsynaptic scaffolding protein SHANK3, have well-studied functions in mature neurons, new evidence indicates that they may subserve novel, distinct roles in immature cells as the nervous system is assembled in prenatal development. Emerging tools and technologies, including single cell sequencing and human cellular models of disease, are illuminating differential isoform utilization, spatiotemporal expression, and subcellular localization of ion channels and synaptic proteins in the developing brain compared to the adult, providing new insights into the regulation of developmental processes. We propose that it is essential to consider the temporally distinct and cell-specific roles of these proteins during development and maturity in our framework for understanding neuropsychiatric disorders.

In Brief

In this Perspective, Panagiotakos and Pașca highlight mechanisms conferring unique developmental roles to disease-associated ion channels and synaptic proteins, arguing that incorporating spatiotemporally-distinct and cell type-specific functions is essential for our emerging framework for understanding neuropsychiatric disorders.

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Introduction

The nervous system builds itself through a series of cellular processes that emerge from an elaborate set of genetic programs, encompassing stem and progenitor cell proliferation to neuronal migration and terminal differentiation (Di Bella et al., 2021; Greig et al., 2013; Leone et al., 2008; Miyata et al., 2010; Rubenstein and Rakic, 1999; Shibata et al., 2015). The coordinated cellular choreography that underlies neural development generates immense cell diversity, incorporating features such as the distant migration of multiple cell types from their place of origin into their final circuit, the elimination of certain cells even after they have been integrated into circuits, and the conserved timing of specific cellular events across different species (Bonnefont and Vanderhaeghen, 2021; Jabaudon, 2017; Kwan et al., 2012; Wamsley and Fishell, 2017; Wong and Marín, 2019). This dynamic selforganization of the developing nervous system often involves utilizing the same gene, gene network, signaling pathway or cellular process in different contexts and time periods. In this perspective, we discuss novel, surprising roles for proteins associated with neurodevelopmental disorders, placing special emphasis on dynamic changes in their expression and regulation that may critically impact disease-related cellular phenotypes. Our focus is specifically on ion channels and synaptic proteins, as they have been strongly linked to neurodevelopmental disorders and proposed as convergence points for disease etiology (De Rubeis et al., 2014; Gilman et al., 2011; Iossifov et al., 2014; Jamain et al., 2003; Levy et al., 2011; Sanders et al., 2011; Zoghbi, 2003). We present selected examples illustrating that temporally regulated and cell-specific changes in gene transcription, alternative splicing and subcellular localization result in the emergence of unique developmental functions of these disease-relevant proteins. Incorporating these roles into efforts towards a systems-level understanding of neurodevelopmental disorders is likely to provide insights into both the proper assembly of the nervous system, as well as the consequences of disease risk-conferring gene variants on neuronal identity and mature circuit function.

Temporally-regulated and cell type-specific enrichment of ion channel subtypes

Precise spatiotemporal regulation of gene expression is a hallmark of normal nervous system development (Miller et al., 2014; Nowakowski et al., 2017; Pletikos et al., 2014; Polioudakis et al., 2019; Telley et al., 2016; Thompson et al., 2014; Trevino et al., 2020; Zhu et al., 2018b), and a number of studies in recent years have brought to light the developmental dynamics of transcripts encoding ion channel subunits, ion pumps and synaptic components (Gordon et al., 2021; Mayer et al., 2019; Smith et al., 2018, 2021; Vitali et al., 2018). Single cell RNA sequencing efforts are also providing unprecedented transcriptomic resolution of neural cell types (Hodge et al., 2019; Yao et al., 2021), allowing for the interrogation of disease-relevant gene expression in specific neural populations (Trevino et al., 2021; Velmeshev et al., 2019; Zuccaro et al., 2021). As ion channels and synaptic proteins have emerged as significant players in neurodevelopmental disorders (De Rubeis et al., 2014; Gilman et al., 2011; Iossifov et al., 2014; Jamain et al., 2003; Levy et al., 2011; Sanders et al., 2011; Zoghbi, 2003), understanding how their expression is regulated across

development both temporally and spatially is essential for our ability to define the temporal windows and candidate cell types that are altered in the context of disease.

A prime example of the phenotypic consequences of temporally-regulated enrichment of disease-associated ion channels is the voltage-gated sodium channels $Na_v1.1$, $Na_v1.2$, and $Na_v1.3$, encoded by *SCN1A*, *SCN2A*, and *SCN3A*, respectively (Smith and Walsh, 2020). Both gain- and loss-of-function mutations have been identified in all three genes encoding these sodium channels and are associated with different developmental phenotypes (Meisler and Kearney, 2005; Meisler et al., 2021; Sanders et al., 2018; Zaman et al., 2020). *SCN3A* expression is elevated in immature progenitors and neurons of the fetal brain, giving way to postnatal expression of *SCN1A* and *SCN2A* (Beckh et al., 1989; Smith et al., 2018). Consequently, emerging data indicates that mutations in *SCN3A* can lead to developmental brain malformations like polymicrogyria (Smith et al., 2018; Zaman et al., 2020), whereas *SCN1A* and *SCN2A* variants typically affect neurophysiology and are more commonly associated with infantile epilepsies (Meisler and Kearney, 2005; Sanders et al., 2018). In line with this, a recent study demonstrated that expression of disease-associated *SCN3A* variants in immature neurons of the developing human and ferret cerebral cortex results in deficits in neuronal migration and placement, in addition to malformed gyri (Smith et al., 2018).

In addition to temporal expression dynamics, Na_v channels have been reported to display cell type-specific enrichment in the developing brain, which may also contribute to distinct phenotypic outcomes when they are mutated. $Na_v1.1$ channels, for example, are predominantly expressed in parvalbumin (PV) cortical interneurons (Yu et al., 2006). Consequently, *Scn1a* loss of function selectively reduces sodium current density in inhibitory interneurons (Yu et al., 2006), and behavioral deficits in an *Scn1a* haploinsufficiency mouse model of Dravet syndrome can be rescued by augmenting GABAergic activity with clonazepam (Han et al., 2012). A knock-in *Scn1a* loss-of-function mouse model also displays early postnatal epilepsy due to a transient impairment of PV interneuron activity (Favero et al., 2018; Ogiwara et al., 2007). Intriguingly, the output of PV interneurons appears to normalize in adulthood, owing to compensatory changes in the distribution of sodium channels in these cells (Favero et al., 2018). This observation underscores another important idea: it is critical to distinguish between initial mechanistic drivers of disease onset and secondary cellular and molecular mechanisms contributing to chronic disease states.

In contrast to $Na_v1.1$, $Na_v1.2$ is enriched in cortical pyramidal neurons (Hu et al., 2009; Spratt et al., 2019), and several studies have described electrophysiological impairments in both developing and mature excitatory neurons resulting from *Scn2a* inactivation (Ben-Shalom et al., 2017; Spratt et al., 2019, 2021; Zhang et al., 2021). A recent study also demonstrated that transcripts encoding $Na_v1.2$ are specifically expressed in both the mouse and baboon in a differentiating population of spiking oligodendrocyte progenitors (Gould and Kim, 2021). This is consistent with previous work suggesting that oligodendrocyte spiking is dependent on $Na_v1.2$ currents (Berret et al., 2017; Jiang et al., 2013). *SCN2A* inactivation disrupts oligodendrocyte maturation (Gould and Kim, 2021), highlighting an emerging, unconventional role for $Na_v1.2$ channels in the differentiation of a cell type not traditionally thought of as “excitable”. Future studies exploring the functional

consequences of individual mutations, as well as their impact on the expression and functions of these channels in specific developing cell populations, are an essential next step towards unraveling the contributions of cell type-specific dysfunction to disease etiology. Understanding how disease-associated changes in electrical signals and ionic influx alter developmental events in human-specific cell types will be important. Towards this goal, approaches functionally characterizing multiple disease-relevant ion channel mutations, at scale and in human cells (e.g. using automated patching, Optopatch), and connecting gene expression with cell function (Patch-seq) will be particularly informative.

Other disease-associated ion channels display dynamic patterns of subunit composition during different developmental windows, including GABA receptor subunit switching during differentiation (Mayer et al., 2019), AMPA receptor subunit switching during neuronal maturation (Kumar et al., 2002), and shifting ratios of voltage-gated potassium channel subunits encoded by *KCNQ2* and *KCNQ3* in early postnatal life (Kanaumi et al., 2008; Tinel et al., 1998). Here we would like to highlight another important developmental switch in ion channel subunit composition: the temporal utilization of NMDA receptor subunits GluN2A and GluN2B, encoded by *GRIN2A* and *GRIN2B*, respectively. Early studies in the embryonic turtle cortex demonstrate that NMDA receptor expression and functional NMDA currents precede the formation of synapses in differentiating neurons (Blanton et al., 1990), and it has been reported that a switch in subunit composition from GluN2B to GluN2A begins in rodent cortical neurons at birth (Sheng et al., 1994; Watanabe et al., 1992). Similarly, studies on human fetal tissue report declining expression of *GRIN2B* as cortical development proceeds (Bagasrawala et al., 2017). This key developmental transition can be recapitulated in human induced pluripotent stem (hiPS) cell-derived cortical organoids that were allowed to mature for extensive periods *in vitro* (Gordon et al., 2021).

Compared to their GluN2B-containing counterparts, GluN2A-expressing NMDA receptors have distinct electrophysiological properties, including more rapid desensitization and different responses to allosteric modulators (Paoletti et al., 2013; Wyllie et al., 2013). As a consequence of the enrichment pattern of channels containing these subunits, *GRIN2B* gene variants are often associated with earlier neurodevelopmental phenotypes, including cortical malformations and autism spectrum disorders (ASD) (Endele et al., 2010; O'Roak et al., 2012). In line with this, inactivation of *Grin2b* in rodent models points to roles for GluN2B-containing NMDA receptors in developmental events occurring during late prenatal and early postnatal life, including migration, morphological maturation and circuit integration (De Marco García et al., 2015; Espinosa and Luo, 2008; Jiang et al., 2015). For example, knocking down GluN2B, but not GluN2A, in the embryonic rodent cortex results in selective deficits in radial migration (Jiang et al., 2015). In developing mouse hippocampal neurons and layer IV cortical neurons, cell-autonomous GluN2B function is also required for proper pruning of primary dendrites and dendritic patterning, respectively, but appears unnecessary for initial dendritic elaboration (Espinosa and Luo, 2008). In the neonatal somatosensory cortex, GluN2B is essential for morphological maturation and circuit integration of specific developing cortical interneuron subtypes (De Marco García et al., 2015). Intriguingly, GluN2B-containing NMDA receptors in these populations are specifically activated by projections from the thalamus, suggesting a potential mechanism by which distinct circuit

components can be selectively affected by disease-associated mutations. Several studies are also illuminating, however, that the dynamics of GluN2 subunit expression may differ in individual cell types (Matta et al., 2013). For instance, whereas medial ganglionic eminence (MGE)-derived interneurons display an activity-dependent GluN2 subunit switch, a subpopulation of caudal ganglionic eminence (CGE)derived hippocampal interneurons retains GluN2B-containing NMDA receptors throughout development, well into the third postnatal week in the mouse (Matta et al., 2013). GluN2B also appears to play a critical role in adult-born neurons as they integrate into olfactory circuits (Kelsch et al., 2012). These findings support the notion that, based on cell type-specific differences in the dynamics of ion channel subunit expression, disease-associated mutations may impinge on different cell types at distinct developmental stages to contribute to the emergence of clinically distinct phenotypes.

Intriguingly, a recent study has implicated rare truncating and damaging missense variants in *GRIN2A* in schizophrenia risk (Singh et al., 2020), highlighting that mutations in different developmentally regulated ion channel subunits may also lead to distinct neuropsychiatric conditions with differing age of onset. Single cell RNA sequencing analysis on human fetal cortical specimens has demonstrated expression of *GRIN2A* in human cortical neural stem cells (Mayer et al., 2019), suggesting a more dynamic pattern of *GRIN2A* regulation through cortical lineage progression. This underscores, once again, the importance of exploring functional consequences of disease-associated *GRIN2* variants at different developmental stages, in different cell types, and in humans as well as other species. Indeed, ion channels are becoming increasingly implicated in normal neural progenitor cell function. Temporally regulated progenitor hyperpolarization, driven in large part by dynamic changes in the expression of potassium channels during embryonic development, is essential for the normal sequential progression of cortical neurogenesis (Vitali et al., 2018). More recently, consistent with a dynamic role for ion channels in neural progenitor cell proliferation, neurodevelopmental disease-associated hyperpolarization activated cyclic nucleotide-gated cation (HCN) channels were shown to regulate progenitor cell cycle dynamics, with HCN loss of function resulting in G1 accumulation and microcephaly (Schlusche et al., 2021). Taken together, these studies and others are defining new roles for ion channels, which have been canonically associated with mature neuronal function, whereby they can regulate progenitor proliferation and other earlier stages of brain assembly.

Another area of growing interest centers around the understanding of evolutionary conservation and divergence of genetic networks underlying neurodevelopmental processes. An extensive transcriptomic study conducted across 16 regions of the developing human and macaque brain recently found that heterochronic processes between the two species include molecular pathways related to synaptic activity (Zhu et al., 2018b). Of note, the genes encoding the ASD-associated synaptic proteins SHANK2 and SHANK3 were expressed earlier in the macaque neocortex and other brain regions relative to humans, whereas the schizophrenia-associated *GRIA1*, encoding the AMPA receptor subunit GluR1, is expressed earlier in the developing human brain (Zhu et al., 2018b). Such studies are beginning to illuminate dynamic species-specific differences in the expression timing of disease-associated transcripts involved in modulating neuronal activity during development,

shedding light both on evolutionary changes in the coordination of developmental programs, as well as on human-specific features of disease-associated gene regulation.

Temporally regulated splicing of ion channels and synaptic proteins

In comparison to other tissues, the brain exhibits substantial levels of alternative splicing (Yeo et al., 2004). In the embryonic brain, dynamic changes in splicing contribute to key developmental transitions (Boutz et al., 2007; Makeyev et al., 2007; Zheng and Black, 2013), including the process of neuronal differentiation in the cerebral cortex (Zhang et al., 2016), and several studies have illuminated developmental splicing switches in genes encoding ion channels or related to synaptic transmission (Flaherty et al., 2019; Gazina et al., 2010; Liang et al., 2021; Panagiotakos et al., 2019; Tang et al., 2009, 2011; Weyn-Vanhentenryck et al., 2018; Zheng et al., 2012). Not surprisingly, given the importance of cell type-specific splicing in dictating key steps in neural development, genome-wide changes in alternative splicing and activity-dependent splicing networks have been reported in neuropsychiatric disorders like ASD, schizophrenia and bipolar disorder (Gandal et al., 2018a; Irimia et al., 2014; Parikshak et al., 2016; Quesnel-Vallières et al., 2016; Sanders et al., 2020; Weyn-Vanhentenryck et al., 2014).

Mutations in individual ion channels or synaptic proteins that impact alternative splicing also play essential roles in the emergence of disease-relevant cellular phenotypes (Flaherty et al., 2019; Panagiotakos et al., 2019). Whereas significant overlap is observed in differential gene expression across neuropsychiatric disorders, changes in cell type-specific isoform expression show a high degree of disease specificity (Gandal et al., 2018a). Disease-specific alterations in splicing machinery (e.g. *RBFOX1*) that impinge on transcripts encoding ion channels and synaptic proteins have also been reported (De Rubeis et al., 2014; Gandal et al., 2018a; Lee et al., 2016; Tang et al., 2009; Weyn-Vanhentenryck et al., 2014). Importantly, activity-dependent splicing factors like Rbfox1 have been shown to direct cell-type specific splicing programs in developing cortical circuits (Wamsley et al., 2018), suggesting another possible molecular substrate for the emergence of distinct cellular phenotypes. These findings demonstrate the necessity of understanding spatiotemporally regulated and cell type-specific splicing patterns in the brain across the lifespan to define cell- and circuit-specific mechanisms underpinning different neuropsychiatric disorders.

The impact of altered splicing in the context of neurodevelopmental disease is exemplified by the monogenic, syndromic ASD Timothy Syndrome (TS). Classical TS is caused by a point mutation in the alternatively spliced *exon 8A* of *CACNA1C*, which encodes the pore-forming subunit of the neuropsychiatric disease-associated calcium channel $Ca_v1.2$ (Bhat et al., 2012; Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Panagiotakos et al., 2019; Paşca et al., 2011; Splawski et al., 2004). *CACNA1C* isoforms containing *exon 8A* are enriched in immature cortical progenitors and young neurons in both the mouse and human, and neuronal differentiation is accompanied by a switch in *CACNA1C* exon utilization from *exon 8A* to its mutually exclusive counterpart *exon 8* (Figure 1) (Panagiotakos et al., 2019; Tang et al., 2011). Intriguingly, in addition to increasing calcium influx by impairing voltage-dependent channel inactivation (Paşca et al., 2011; Splawski et al., 2004), the TS mutation prevents the splicing switch in *CACNA1C*

from *exon 8A* to *exon 8* in differentiating human cortical excitatory neurons from TS patients (Panagiotakos et al., 2019). Consequently, TS cortical neurons derived from hiPS cells continue to express *exon 8A* transcripts containing the mutation at increased levels. This prolonged developmental misexpression of mutant channels later in development and the ensuing reduction in wild type *exon 8*-expressing channels contribute to impairments in the differentiation of deep layer cortical projection neurons (Panagiotakos et al., 2019; Paşca et al., 2011), which have been implicated in the pathogenesis of ASD (Willsey et al., 2013).

In addition to differentiation deficits, TS channels have been linked to phenotypes in different cell types and at different stages of cortical neuron maturation, including impaired interneuron migration (Birey et al., 2017), deficits in radial migration in developing excitatory neurons (Kamijo et al., 2018)dendritic retraction in cortical pyramidal neurons (Krey et al., 2013). Investigating how altered splicing contributes to these developmental phenotypes underlying TS in different neural cell types is thus of great interest. It should be noted here that since *CACNA1C* transcripts differing only in *exon 8* or *exon 8A* expression generate $Ca_V1.2$ channels with only subtle differences in channel physiology (Welling et al., 1997; Zühlke et al., 1998), it is possible that swapping these exons alone may predominantly impact channel conformation or downstream signaling interactions to affect neuronal development, as opposed to simply changing ionic flux. Further supporting a role for the TS mutation in promoting splicing deficits in *CACNA1C*, we recently discovered using hiPS cell-derived forebrain assembloids that the splicing switch from *exon 8A* to *exon 8* is also impaired in interneuron populations from TS patients (Birey et al., 2021).

Developmental, cell- and tissue-specific regulation of other *CACNA1C* exons has been reported in the human, as well as the rodent, (Diebold et al., 1992; Soldatov, 1994; Tang et al., 2007, 2009; Welling et al., 1997), with a recent study identifying multiple fulllength region-specific *CACNA1C* splice isoforms in the adult human brain (Clark et al., 2020). As inclusion of some exons can significantly impact channel properties (Tang et al., 2009), it is likely that alternative splicing plays a key role in directing the developmental functions of these channels by generating isoforms with distinct biophysical and/or signaling properties. Perhaps disease-causing mutations cause specific deficits by influencing splicing to generate specific isoforms during discrete developmental windows. Incorporating the expression patterns of different channel splice variants into our framework for understanding *CACNA1C*-associated neuropsychiatric disorders will illuminate the cell types or developmental stages that drive the emergence of specific disease-relevant cellular phenotypes.

Other disease-associated ion channels also display developmentally regulated splice isoform utilization (Gazina et al., 2015; Liang et al., 2021; Liu et al., 2019; Plummer et al., 1997; Smith et al., 2001). For example, *SCN2A*, the gene encoding the voltage-gated sodium channel $Na_V1.2$, is strongly associated with ASD, intellectual disability (ID) and infantile seizures (Sanders et al., 2018). *SCN2A* contains two mutually exclusive exons, *5N* and *5A*, whose utilization is dynamically controlled during development and into adulthood (Gazina et al., 2015; Liang et al., 2021). The “neonatal” *5N* exon is enriched in *SCN2A* transcripts in the embryonic and early postnatal brain (Gazina et al., 2015; Liang et al., 2021). In rodents, *5N* inclusion declines postnatally as *Scn2a* mRNA levels increase, resulting in “adult” exon

5A-containing isoforms encoding the majority of $Na_V1.2$ channels in cortical neurons after the second postnatal week (Gazina et al., 2010, 2015). These splicing dynamics also differ across brain regions in the mouse (Gazina et al., 2010), and different patterns of *exon 5A* and *5N* splicing have been reported in the genes coding for other voltage-gated sodium channel alpha subunits in the mouse and human brain, including the neurodevelopmental disease-associated *SCN8A* (encoding $Na_V1.6$) (Gazina et al., 2010; Liang et al., 2021).

Expression of *exon 5A* or *5N*-containing *SCN2A* variants in cortical excitatory neurons is predicted to differentially impact channel properties and thereby neural excitability. For instance, neonatal isoforms result in reduced neuronal excitability (Gazina et al., 2015; Xu et al., 2007). A recent report also suggests that cell type-specific *SCN2A* splice variants may generate channels that are optimized for the electrophysiological properties of the neurons in which they are expressed (Lignani et al., 2020). Notably, studies have found that multiple *SCN2A* mutations associated with infantile epilepsy preferentially or more strongly impact channels encoded by neonatal *5N* isoforms, rendering neonatal channels more functionally similar to adult channels (Thompson et al., 2020; Xu et al., 2007). Other data indicate that splice isoforms of sodium channels associated with epilepsy disorders, such as $Na_V1.1$ (encoded by *SCN1A*), are differentially responsive to anti-epileptic drugs (Tate et al., 2005; Thompson et al., 2011). This suggests that alternative splicing also has important consequences for developing therapies. Collectively, these findings underscore that it is essential to profile the expression of specific ion channel splice variants across development and to functionally characterize their impact on different developmental processes. This will enable the field to better predict the consequences of disease-causing mutations and the effects of therapeutic interventions.

Looking ahead, as with Timothy Syndrome, the use of hiPS cell-derived organoid and assembloid platforms will be especially powerful to probe the functional consequences of *SCN2A* splice isoforms and disease-associated mutations in human cells (Paşca, 2018). Human neurons lacking one or both copies of *SCN2A* have been generated from control hiPS cells or human embryonic stem (hES) cell lines using CRISPR approaches and have been shown to phenocopy some of the electrophysiological phenotypes reported in *Scn2a* mouse models (Deneault et al., 2018; Lu et al., 2019; Ogiwara et al., 2018; Planells-Cases et al., 2000). To date, hiPS cell derivatives have been successfully used to probe cellular phenotypes resulting from *SCN1A* mutations in Dravet syndrome patients (Jiao et al., 2013; Liu et al., 2013; Sun et al., 2016).

In addition to ion channels, synaptic proteins are known to undergo alternative splicing to generate cell type-specific isoforms, as exemplified by the presynaptic neurexins (Chih et al., 2006; Dai et al., 2019; Fuccillo et al., 2015; Jenkins et al., 2016; Schreiner et al., 2014; Treutlein et al., 2014). *Nrxn1*, for example, is alternatively spliced to yield an array of cell type-specific neurexins in the mouse cerebral cortex that specify individual synapses by interacting selectively with isoforms of different post-synaptic partners (most notably the neuroligins) (Fuccillo et al., 2015; Schreiner et al., 2014). In humans, deletions in *NRXN1* (encoding neurexin-1) have been strongly associated with various neuropsychiatric disorders, including ASD and schizophrenia (Ching et al., 2010; De Rubeis et al., 2014; Kim et al., 2008), and differential splicing of *NRXN1* has been reported in ASD, schizophrenia

and bipolar disorder (Gandal et al., 2018a). *NRXN1* expression and *NRXN1α* and *NRXN1β* isoform levels have been shown to increase during fetal development and peak at birth in post-mortem human cortical specimens (Jenkins et al., 2016), and a recent study reports that *NRXN1α* isoform diversity is developmentally regulated, with fetal prefrontal cortex samples displaying substantially higher isoform diversity than both adult prefrontal cortex and hiPS cell-derived control neurons (Flaherty et al., 2019). This latter study goes on to show that the abundance of wild type *NRXN1α* isoforms is markedly reduced in patient-derived neurons bearing heterozygous loss-of-function mutations in *NRXN1*, accompanied by increased expression of *NRXN1β* isoforms and, in some genotypes, aberrant expression of novel "mutant" *NRXN1α* splice variants (Flaherty et al., 2019). Changes in *NRXN1* splice isoform expression resulting from *NRXN1* mutations were correlated with deficits in neuronal activity, morphology and maturation (Flaherty et al., 2019). Intriguingly, overexpression of wild type *NRXN1α* isoforms was not able to rescue patient neurons that expressed dominant negative "mutant" *NRXN1α* isoforms, highlighting the significance of understanding the precise impact of different disease-causing mutations on the splice variant repertoire of synaptic proteins and the pathological consequences of altered splicing on neuronal function. Functional studies in rodents indicate that combinatorial transsynaptic interactions between specific pairs of presynaptic neurexin and postsynaptic neuroligin isoforms are critical for synaptic assembly, plasticity and cell-specific connectivity (Singh et al., 2016; Uchigashima et al., 2020). Disease-associated mutations in *NRXN1* could potentially eliminate *NRXN1* isoforms that specify individual synaptic types, or, as described by Flaherty et al., could introduce "mutant" isoforms that alter the repertoire of *NRXN1* isoforms to ultimately influence neuronal activity (Flaherty et al., 2019). How such "mutant" isoforms interfere with synaptic function or whether they take on novel roles remains unclear. Mutations in genes encoding astrocyte-secreted linker proteins that enable interactions between "incompatible" neurexin-neuroligin isoform pairs (Singh et al., 2016) could also indirectly impinge on neurexin signaling. One such protein, encoded by *SPARCL1*, has been associated with ASD (De Rubeis et al., 2014). Given the cell type specificity of neurexins, it will be especially important to interrogate the impact of *NRXN1* mutations in different cell types and at different developmental stages.

In summary, two key ideas are essential to consider in our discussion of mechanisms that contribute to the emergence of specific cellular phenotypes in neurodevelopmental disorders. Our knowledge of the spatiotemporal expression profiles and unique functions of ion channel and synaptic protein isoforms will enable a deeper understanding of how mutations that confer risk for neurodevelopmental disorders can lead to circuit dysfunction. At the same time, some disease-relevant mutations may promote misexpression of splice variants at inappropriate times or in the incorrect cell types, or alternately, aberrant expression of "mutant" splice isoforms, to drive cellular phenotypes that underlie disease.

Developmentally regulated subcellular localization

In addition to developmentally regulated, cell-specific gene expression and alternative splicing events, temporal changes in subcellular localization may represent another key contributor to the unique developmental functions of disease-related ion channels and synaptic proteins. As mentioned above, loss-of-function mutations in *SCN2A* have been

associated with ASD and ID, whereas gain-of-function *SCN2A* variants have been linked to infantile epilepsies (Sanders et al., 2018). While only a small fraction of *SCN2A* disease-associated mutations have been functionally characterized, a recent study demonstrated that 11 different ASD-associated *SCN2A* variants inhibited or completely abrogated $Na_v1.2$ channel function in HEK cells. Moreover, these mutations had more pronounced effects on the excitability of immature cortical neurons as compared to mature pyramidal cells (BenShalom et al., 2017). While it is possible that the temporal switch from *5N*-expressing *SCN2A* variants to adult *5A* isoforms contributes to the selective consequences of these ASD mutations in immature neurons, the specificity of these deficits likely also results from the developmental regulation of $Na_v1.2$ subcellular localization during early postnatal life.

In immature cortical neurons, $Na_v1.2$ channels are expressed throughout the axon and in the axon initial segment (AIS), making them critical for action potential generation and propagation (Figure 2) (Boiko et al., 2003; Gazina et al., 2015; Spratt et al., 2019). In mature cortical neurons, however, $Na_v1.2$ channels are replaced by $Na_v1.6$ at the distal AIS and nodes of Ranvier, and the restriction of $Na_v1.2$ expression to the proximal AIS, soma and dendrites changes its contribution to mature neuronal function (Figure 2) (Boiko et al., 2003; Gazina et al., 2015; Hu et al., 2009; Spratt et al., 2019). As a consequence of this developmental reorganization of $Na_v1.2$ channel distribution, $Na_v1.2$ loss of function has differential effects on cortical neurons at different stages of maturation: whereas *Scn2a* haploinsufficiency in immature neurons during the first postnatal week reduces neuronal excitability and impairs spiking, mature *Scn2a*$^{+/-}$ cortical neurons instead display deficits in dendritic action potential backpropagation and synaptic function, as well as structurally immature dendritic spines (Spratt et al., 2019). Two recent studies further interrogated the effects of complete *Scn2a* loss of function on mature neuronal activity, discovering unexpected cellular phenotypes upon complete inactivation of *Scn2a* (*Scn2a*$^{-/-}$) (Spratt et al., 2021; Zhang et al., 2021). Notably, adult mice lacking both copies of *Scn2a* in mature cortical neurons display increased neuronal excitability due to compensatory changes in potassium channel expression (Spratt et al., 2021; Zhang et al., 2021). These tantalizing data set the stage for future studies exploring: how different *SCN2A* variants impact dendritic structure, neuronal excitability and synaptic function at specific stages of neuronal maturation; how early changes in neuronal maturation contribute to circuit assembly and function; how compensatory changes in ion channel expression or distribution can result in paradoxical phenotypes; and how the developmental change in $Na_v1.2$ channel localization contributes to the phenotypic heterogeneity of *SCNA*-associated disorders.

Recent studies have also shed light on spatiotemporal regulation of synaptic protein distribution as a potential driver of their novel developmental functions. In one study, the ASD and Phelan-McDermid (PMD) Syndrome-associated synaptic protein SHANK3 (Bonaglia et al., 2001; De Rubeis et al., 2014; Durand et al., 2007; Sanders et al., 2015; Satterstrom et al., 2020) was found to play a critical but unexpected developmental role in regulating intrinsic neuronal excitability in human neurons (Yi et al., 2016). SHANK3 is enriched in neuronal post-synaptic densities, where it acts as a scaffold that orchestrates post-synaptic signaling (Naisbitt et al., 1999; Sheng and Kim, 2000). Consequently, it has been thought that mutations affecting SHANK3 expression or function cause neurodevelopmental deficits primarily by impairing synaptic activity (Bozdagi et al.,

2010; Kouser et al., 2013; Monteiro and Feng, 2017; Wang et al., 2011). Interestingly, however, PMD patient neurons derived from hiPS cells display increased input resistance in addition to altered synaptic transmission (Kathuria et al., 2018; Shcheglovitov et al., 2013), supporting the possibility that SHANK3 may subserve a distinct earlier role separate from its conventional function as a scaffolding protein at the synapse. Using homologous recombination to conditionally inactivate *SHANK3* in hES cell lines, Yi et al. generated matched isogenic control and *SHANK3* mutant neurons and found that *SHANK3* loss of function, in addition to causing synaptic deficits, resulted in preceding changes: impaired dendritic arborization and increased neuronal input resistance and excitability (Yi et al., 2016). The authors show that this effect on neuronal excitability is mediated by a direct interaction between SHANK3 and voltage gated HCN channels. They also demonstrate that persistent pharmacological suppression of I_h currents phenocopies the deficits in neuronal morphology and synaptic function observed in *SHANK3* mutant neurons. This novel, earlier developmental function of SHANK3 may thus underlie the constellation of cellular phenotypes resulting from *SHANK3* disease-associated loss-of-function mutations.

The role of SHANK3 in regulating neuronal excitability is also highlighted by a recent assembloid model of the cortico-striatal circuit (Miura et al., 2020). Striatal organoids derived from patients with PMD did not display changes in neuronal activity when grown in isolation, as measured by calcium imaging, but following assembly with cortical glutamatergic neurons, striatal neurons from PMD patients displayed an increased number of calcium spike events (Miura et al., 2020). These data in human cells are consistent with a study in rodents demonstrating that early increases in cortical activity in *Shank3B*$^{-/-}$ mutant animals drive premature maturation of striatal projection neurons and corticostriatal connectivity (Peixoto et al., 2016). A subsequent study performed in *Shank3B*$^{-/-}$ mice revealed that cortical hyperactivity is the result of impaired interneuron function, and selective disruption of *Shank3* in cortical interneurons promotes pyramidal neuron hyperexcitability (Chen et al., 2020). These findings reinforce the importance of interrogating the function of neuropsychiatric disease risk genes in different cell types to uncover disease mechanisms.

Notably, transcripts encoding proteins in the SHANK family are alternatively spliced to generate isoforms with distinct subcellular localization and temporal expression patterns in the mouse (Lim et al., 1999; Peça et al., 2011; Wang et al., 2014) suggests that differential expression of *SHANK3* isoforms may also contribute to diverse subcellular functions of SHANK3 proteins. While *Shank3B*$^{-/-}$ mice completely lack two of the major *Shank3* isoforms and express markedly reduced levels of a third isoform (Peça et al. 2011), mouse models in which different *Shank3* isoforms are deleted have been generated by targeting regions corresponding to specific protein interaction domains (Zhu et al. 2018). Such models also support the idea that *Shank3* deficiency impacts neuronal excitability (Zhu et al., 2018a). However, only disruption of specific *Shank3* isoforms is associated with HCN channelopathy, whereas disrupting a domain that spares one isoform does not impact I_h currents (Zhu et al., 2018a). Understanding how neurodevelopmental disease-associated mutations in *SHANK3* impinge on the subcellular localization and function of individual *SHANK3* isoforms in different neuronal subtypes will therefore be essential to shed light on the unique cellular phenotypes resulting from different mutations (Wang et al., 2014).

In a similar vein, the gene encoding the ASD and ID-related synaptic protein SynGAP, *SYNGAP1*, is alternatively spliced to generate proteins that display unique spatiotemporal localization in immature neurons (Araki et al., 2020). SynGAP is a postsynaptic protein localized to dendritic spines that has been shown to interact with PSD-95 and regulate synaptic plasticity (Chen et al., 1998; Kim et al., 1998). Multiple ASD- and ID-associated *de novo* splice site mutations in *SYNGAP1* have been identified (Vlaskamp et al., 2019), and loss-of-function *SYNGAP1* variants have been strongly associated with a variety of neurodevelopmental disorders (Deciphering Developmental Disorders Study, 2015; Hamdan et al., 2009; Parker et al., 2015; Rauch et al., 2012). A recent study by Araki et al. demonstrates differential subcellular localization and biochemical properties of distinct SynGAP isoforms. Expression of the shorter SynGAP-β isoform, which is diffusely cytoplasmically localized in immature neurons, precedes expression of synaptically targeted SynGAP isoforms and plays a specific role in the elaboration of dendritic arbors (Figure 3) (Araki et al., 2020). In contrast, the most well studied SynGAP isoform, SynGAP-α1, is concentrated at the postsynaptic density by virtue of two structural domains, both of which SynGAP-β lacks: a coiled-coiled domain enabling phase separation and a PDZ binding domain enabling tight association with PSD-95 (Araki et al., 2020). Mutations in SynGAP-α1 preventing phase separation or PSD-95 binding render it more similar to SynGAP-β, disrupting synaptic targeting and redistributing SynGAP-α1 to the dendrite, where it then contributes to dendritic arborization (Araki et al., 2020). This work underscores the importance of characterizing the spatiotemporal expression patterns and sub-cellular functions of different isoforms in order to inform our understanding of neurodevelopmental disease-associated *SYNGAP1* variants.

hiPS cell-derived neurons engineered to lack all SynGAP isoforms display alterations in dendritic morphogenesis and synaptic transmission (Llamosas et al., 2020) It remains unclear, however, how different SynGAP isoforms are localized to regulate distinct aspects of neuronal maturation in human cells and how these isoforms might be disrupted by disease-associated *SYNGAP1* mutations. Together, these examples draw attention to dynamic changes in the subcellular localization of disease-relevant ion channels and synaptic proteins that can give rise to distinct functions in different cellular compartments and at different stages of development. They reinforce the need to conduct parallel investigations in model organisms and human cells using single cell and spatial “omics” approaches to probe the dynamic spatiotemporal and cell type-specific changes that occur at critical developmental transitions.

Looking ahead

A burgeoning of genetic studies has identified many neuropsychiatric disease-associated risk variants. Substantial evidence has also emerged pointing to genetic overlap across multiple neuropsychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013; Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2013; Cross-Disorder Group of the Psychiatric Genomics Consortium. Electronic address: plee0@mgh.harvard.edu and Cross-Disorder Group of the Psychiatric Genomics Consortium, 2019; Gandal et al., 2018b). The use of integrative analyses on genetic and proteomic datasets in the past decade has defined splicing networks, protein interaction

maps, and neurodevelopmental programs onto which genetic risk converges (Neale et al., 2012; O'Roak et al., 2012; Sanders et al., 2012; Sullivan and Geschwind, 2019). More recently, bulk and single cell interrogation of chromatin dynamics and gene regulatory networks in the developing human cortex has enabled the identification of novel ASD-associated gene variants and the mapping of non-coding mutations in an ASD patient cohort onto specific developing cell types (Markenscoff-Papadimitriou et al., 2020; Trevino et al., 2021). Stemming from such studies, it has been proposed that specific cellular processes, developmental windows or cell types (Gilman et al., 2011; Gulsuner et al., 2013; Li et al., 2018; Parikshak et al., 2016; State and Šestan, 2012; Tebbenkamp et al., 2014; Willsey et al., 2013; Zoghbi, 2003) in the prenatal brain are preferentially vulnerable to neuropsychiatric disease-associated mutations. These ideas have spurred exciting, largescale collaborative efforts aimed at massively parallel interrogations of disease risk genes (Sanders et al., 2019; Willsey et al., 2018, 2021). The ultimate goal of such endeavors is to define gene and protein interaction networks and identify points of convergence at the cellular or circuit level in the context of disease.

It has become increasingly clear, however, that disease-associated risk genes can display dynamic expression trajectories across development (Gulsuner et al., 2013; Li et al., 2018; Tebbenkamp et al., 2014; Willsey and State, 2015; Zhu et al., 2018b). More recent studies are also revealing a complexity beyond the level of gene transcription. As we discuss in this perspective, mechanisms including splice isoform selection and differential subcellular localization confer non-canonical, temporally distinct roles to proteins encoded by disease risk genes. We presented individual examples of spatiotemporal changes in gene expression, isoform utilization and subcellular localization as three axes of dynamic change that bestow novel developmental functions on individual well-studied, disease-associated ion channels and synaptic proteins. These snapshots highlight the possibility that preventing or altering such spatiotemporally regulated events is another way in which disease-associated mutations might disrupt specific cellular behaviors in the developing brain.

As described above, rigorous functional interrogation of disease-associated ion channels and synaptic proteins in differentiating and/or maturing cells is also uncovering unexpected disease-relevant phenotypes. Such cellular phenotypes appear to result as a consequence of the complex spatial, temporal and cell type-specific regulation of these proteins, in some cases secondary to homeostatic mechanisms. To fully understand the contribution of neuropsychiatric disorder risk genes to cellular phenotypes underlying disease, we must extend our emerging systems-level framework of neuropsychiatric disorders. We propose that this should include a more refined mechanistic understanding of how the protein products of disease risk genes dynamically change in space and time within specific cell types to impart unique functions. How do these molecular dynamics in turn impact the association of these proteins with proteins encoded by other risk genes in different neural cell types and at different developmental stages? Moving forward, we must consolidate a more complete understanding of the full repertoire of developmental changes in ion channel and synaptic protein expression and localization, not just individual changes across a single axis as those described above. How are these dynamics regulated across neuronal maturation? In graded or restricted areal- or laminar-specific patterns? With cell type type-specific enrichment? In distinct brain regions? Across lineage progression? How can we

map cellular phenotypes and candidate disease-relevant molecular pathways onto multiple changing trajectories across space and time? Moreover, we must begin to incorporate how extrinsic features, including electrical activity, immune state, and metabolic function, can intersect with these cellular and subcellular properties to promote phenotypic heterogeneity.

ACKNOWLEDGEMENTS

We thank T. Palmer, M. State and J. Rubenstein for critical feedback on our manuscript. We also thank the members of our laboratories for important discussions surrounding disease mechanisms and cell type specificity.

This work was supported by the UCSF Program for Breakthrough Biomedical Research, Sandler Foundation (Faculty Fellows Program), NIH R01 MH125004 and R56 MH127075 (to G.P.), and the Stanford Brain Organogenesis (Wu Tsai Neurosciences Institute), Bio-X, a New York Stem Cell Foundation–Robertson Investigator Award and Chan Zuckerberg Ben Barres Investigator Award, NIH R01 MH115012 (to S.P.P.).

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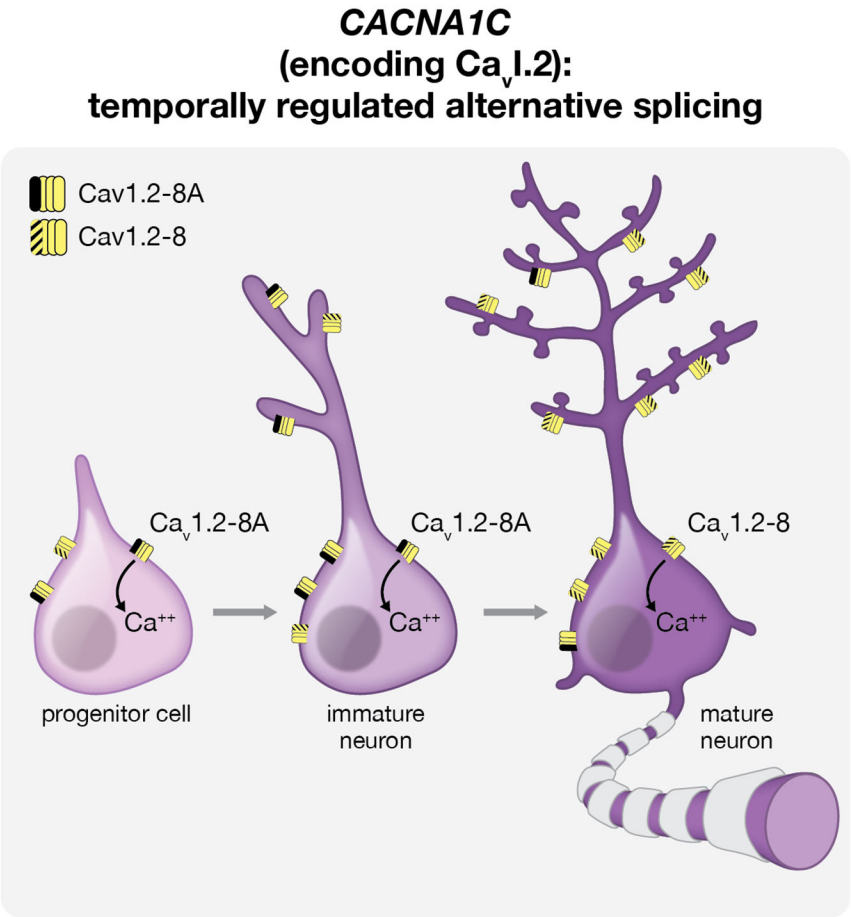

Figure 1:

Dynamic changes in *CACNA1C* splicing may underlie novel roles for $Ca_v1.2$ channels in neuronal differentiation. Utilization of the Timothy syndrome-associated *exons 8* and *8a* of *CACNA1C* in the developing brain is temporally regulated. *Exon 8a*-expressing transcripts are enriched in neural progenitors and immature neurons (**left, center**), whereas channels encoded by *exon 8*-containing transcripts are more highly expressed in mature neurons (**right**). The TS mutation promotes persistently increased expression of *exon 8A* transcripts containing the mutation during neuronal differentiation and maturation.

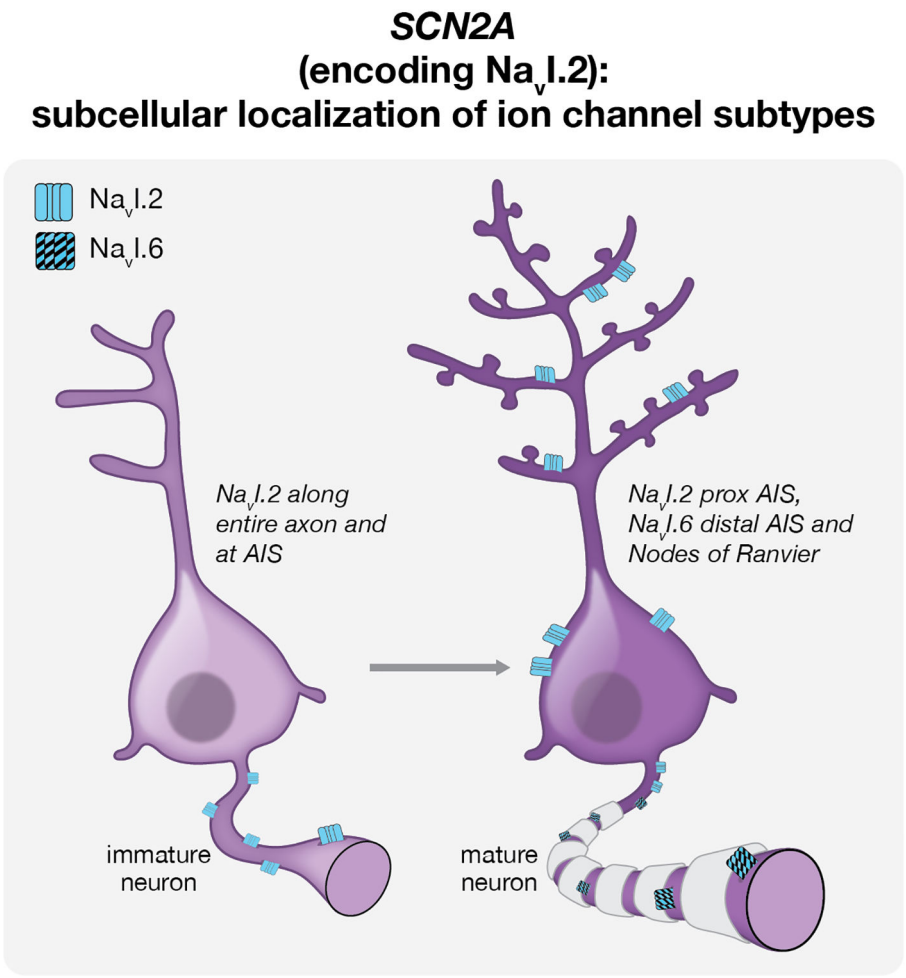

Figure 2:

Developmental reorganization of $Na_v1.2$ channels contributes to distinct functions at different stages of neuronal maturation. In immature neurons (**left**), $Na_v1.2$ channels decorate the axon initial segment (AIS) and entire length of the axon, underpinning their indispensable role in action potential generation and propagation at early postnatal ages. In mature neurons (**right**), $Na_v1.2$ channels display somatodendritic localization and are restricted to the proximal AIS. They are replaced in the distal AIS and Nodes of Ranvier by $Na_v1.6$ channels. As a consequence of this dynamic subcellular regulation of channel distribution, *Scn2a* inactivation yields distinct electrophysiological phenotypes in immature and mature cortical projection neurons, and *SCN2A* loss-of-function variants preferentially affect immature neuron excitability.

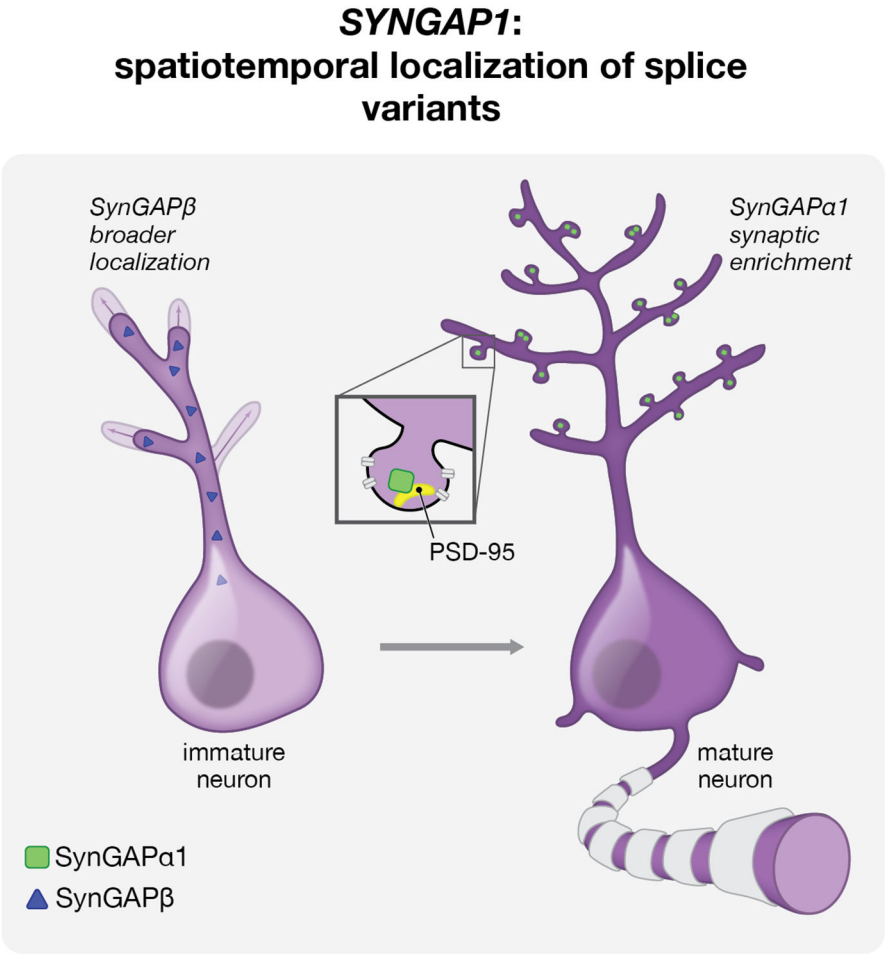

Figure 3:

Alternative splicing of *Syngap1* gives rise to SynGAP isoforms with distinct, temporally-regulated subcellular localization in immature and mature neurons. Earlier and less synaptically-enriched expression of the β isoform of SynGAP in immature neurons (**left**) results in a specific role for this classical synaptic protein in dendritic arborization. In mature neurons (**right**), SynGAP-α1 is enriched at the postsynaptic density, where it directly interacts with PSD-95 and regulates synaptic plasticity. Dendritic arborization is altered in human neurons lacking all *SYNGAP1* isoforms, but it is not yet clear in human cells how this developmental reorganization of SynGAP isoforms might be influenced by *SYNGAP1* mutations.