Early role for a Na⁺,K⁺-ATPase (ATP1A3) in brain development

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Osmotic equilibrium and membrane potential in animal cells depend on concentration gradients of sodium (Na⁺) and potassium (K⁺) ions across the plasma membrane, a function catalyzed by the Na⁺,K⁺-ATPase α-subunit. Here, we describe ATP1A3 variants encoding dysfunctional α-subunits in children affected by polymicrogyria, a developmental malformation of the cerebral cortex characterized by abnormal folding and laminar organization. To gain cell-biological insights into the spatiotemporal dynamics of prenatal ATP1A3 expression, we built an ATP1A3 transcriptional atlas of fetal cortical development using mRNA in situ hybridization and transcriptomic profiling of ∼125,000 individual cells with single-cell RNA sequencing (Drop-seq) from 11 areas of the midgestational human neocortex. We found that fetal expression of ATP1A3 is most abundant to a subset of excitatory neurons carrying transcriptional signatures of the developing subplate, yet also maintains expression in nonneuronal cell populations. Moving forward a year in human development, we profiled ∼52,000 nuclei from four areas of an infant neocortex and show that ATP1A3 expression persists throughout early postnatal development, most predominantly in inhibitory neurons, including parvalbumin interneurons in the frontal cortex. Finally, we discovered the heteromeric Na⁺,K⁺-ATPase pump complex may form nonredundant cell-type-specific αβ isoform combinations, including α3-β1 in excitatory neurons and α3-β2 in inhibitory neurons. Together, the developmental malformation phenotype of affected individuals and single-cell ATP1A3 expression patterns point to a key role for α3 in human cortex development, as well as a cell-type basis for pre- and postnatal ATP1A3-associated diseases.

cortex development | ATP1A3 | developmental channelopathy | polymicrogyria | cortical malformation

Generating a folded, six-layered cerebral cortex relies on the integration of signals from a variety of cell types, coordinating schedules of cell proliferation, migration, differentiation, and maturation. These processes require proper maintenance of ionic gradients and cell membrane potential (Vm) and thus rely on the precise function of ion channels and pumps. Pathogenic mutations in genes encoding ion channels can cause malformations of cortical development (MCDs), collectively known as prenatal developmental channelopathies (1). For example, pathogenic variants in prenatally expressed genes encoding glutamate (GRIN2B, GRIN1) and sodium channels (SCN3A) can lead to MCDs (2–4), at least in part via gain-of-function, cell-autonomous effects in neurons and other cell types (4). These developmental channelopathy variants in glutamate and sodium channels cause pathogenic increases in cationic flux into cells (1), likely affecting Vm in neural progenitor cells (NPCs) and neurons, a perturbation shown to be sufficient to disrupt neurogenic and cortical laminarization processes in experimental models (5, 6).

Neuronal physiology, homeostasis, and signaling depend on the electrogenic activity of the Na⁺,K⁺-ATPase, an ionic pump powered by ATP hydrolysis, which maintains sodium (Na⁺) and potassium (K⁺) gradients across the plasma membrane. The Na⁺,K⁺-ATPase complex contains a large catalytic subunit (α) and two auxiliary subunits (β, FXYD) (7). In vertebrates, paralogous genes (ATP1A1 to -3) encode the brain enriched α-subunits and the ATP1A3 isoform specifically is critical for recovery of electrochemical gradients following neuronal excitation, among several other physiological functions (8–10). Pathogenic variants in ATP1A3 have been increasingly associated with a spectrum of neurological diseases, with phenotypes ranging broadly in age of onset from the immediate postnatal period throughout adulthood.

Significance

By evaluating children with a malformed cerebral cortex, we identified an ATPase pump (ATP1A3) with an early role in brain development. The ATP1A3 pump maintains the physiological concentration of sodium and potassium ions in cells, a process critical for osmotic equilibrium and membrane potential across several developing cell populations. We employed single-cell sequencing approaches to identify key enrichments for ATP1A3 expression during human cortex development. Unravelling this early cell-type-specific pathophysiology in the developing brain offers a potential basis for the treatment of ATP1A3-related diseases affecting prenatal and early childhood development.


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(11, 12), suggesting shifting vulnerabilities to different ATP1A3 dysfunction during the several stages of childhood brain maturation. These diseases include (in approximate order of onset in childhood development): early infantile epileptic encephalopathy (EIEE); alternating hemiplegia of childhood (AHC); cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss (CAPOS); childhood onset schizophrenia; and rapid-onset dystonia-parkinsonism (RDP) (11, 12). Variants in ATP1A3 associated with these postnatal diseases are generally categorized as heterozygous loss-of-function (LOF) single-nucleotide variants (7, 13) that, among other deficits, have been shown to lead to depolarized Vm, such as in neurons from AHC patients (14). This finding, along with findings that bioelectric changes during circuit development can dramatically alter both intrinsic trajectories of individual cells (15) and cortical lamination more broadly (5, 6), suggests that bioelectric alterations due to ATP1A3 dysfunction during embryonic development could in part contribute to an early pathophysiology for both pre- and postnatal-associated ATP1A3-related neurological disorders.

Here, we present four individuals with novel de novo ATP1A3 variants, resulting in polymicrogyria (PMG, the most common brain malformation) (16), epilepsy, and global developmental...
delay, and consider a previously described individual report of AHC with PMG (17). Unlike ATP1A3 variants associated with other conditions, the ATP1A3 variants associated with PMG clustered in a “hotspot” between transmembrane regions 7 and 8 (TM7 and TM8) of the α3 protein. Using mRNA in situ analysis and single-cell RNA sequencing (scRNA-seq, Drop-seq) in the human fetal cortex at midgestation, we show that ATP1A3 is most highly expressed in subset of deep-layer neurons located in the subplate (SP), and is also expressed in intermediate progenitors and neurons. We then show that the fetal cortex also displays a rostral-to-caudal ATP1A3 gradient within SP excitatory neurons (ENs), with ATP1A3 expression levels highest in the prefrontal cortex. Moreover, using Drop-seq in the human infant cortex, we show that postnatal expression of ATP1A3 at this stage of development extends to all EN subtypes (with deeper layer cortical layer biases) and mostly predominates within cortical interneurons, most abundantly enriched to parvalbumin (PV) interneurons in the prefrontal cortex. Finally, within individual cells, variable levels of ATPase α- and β-subunit isoforms offers differential vulnerability between ENs and inhibitory neurons (INs), and may provide a partial explanation for the broad phenotypic range associated with distinct ATP1A3 variants that affect different biological maturation processes within the pre- to postnatal period.

Results

Individuals with ATP1A3 Variants Display Cortical Malformation Phenotypes. MRI of four unrelated individuals with de novo ATP1A3 variants revealed a range of PMG severity, from extensive bilateral frontoparietal PMG to unilateral PMG (Fig. L4). Detailed descriptions of each case are included in SI Appendix, Clinical Descriptions.

Case A: Bilateral frontoparietal PMG (p.Arg901Met). Case A, a female child of nonconsanguineous parents from Portugal, was born at full term and had a short postnatal hospital stay for respiratory distress and jaundice. Brain MRIs performed at 5 mo and at 9 y of age revealed extensive bilateral perisylvian PMG involving the lateral temporal lobes, insulae, and posterior half of frontal and parietal lobes (Fig. L4). The posterior part of the frontal and temporal cortex appears abnormally thick, with somewhat shallow sulci, giving a pachygyric appearance (bilateral and fairly symmetric over the most left-handed individuals), The most anterior aspects of the frontal and parietal lobes, as well as the medial and inferior cerebral cortex, were spared. At 9 y she developed seizures, manifesting as focal impaired awareness, and was treated with oxcarbazepine. She was developmentally and cognitively delayed, able to sit alone and walk with a walker, point and make some signs, but had no speech and could hold a pencil but not write. Asymmetric spastic quadriaparesis (left more significant than right) with hypotonia and athetoid movements of the hands and fingers were noted on examination, as were a high palate, thin upper lip, and smooth philtrum. Her head circumference at 9 y was 51 cm (−0.73 SD), and she exhibited drooling and had feeding difficulties. Trio whole-exome sequencing (WES) revealed a de novo ATP1A3 variant, c.2702G > T (p.Arg901Met), absent in both parents (Fig. 1 and SI Appendix, Fig. S1).

Case B: Extensive bilateral PMG (c.2921+1G > A). Case B, a male child of nonconsanguineous parents from the Philippines, was born at 38-wk gestation. His head circumference at birth was 34 cm (−0.83 SD) and he developed seizures 12 h after delivery. Brain MRI at 6 wk revealed extensive bilateral PMG, and small, cyst-like areas were noted within the hippocampal heads and bodies. Video electroencephalograms (EEGs) confirmed a severe epileptic encephalopathy resistant to essentially all antiepileptic drugs. EEGs at 2 mo of age showed abundant electroclinical and electrogrographic seizures beginning predominantly from the right parietal region but also from the left and bilateral parasagittal regions. Visual and auditory evoked potential studies were normal at 4 and 5 mo of age, respectively. Evaluation at 14 mo revealed microcephaly, with a head circumference of 41.5 cm (−4.63 SD), severe global developmental delay (never rolled over, sat up, or talked), intermittent myasthenia, significant axial and appendicular hypotonia, and hyporeflexia. Trio WES revealed a de novo ATP1A3 variant, c.2921+1G > A, disrupting a conserved splice site, absent in both parents (Fig. L4 and SI Appendix, Fig. S1).

Case C: Unilateral PMG (p.Leu924Pro). Case C, a male child of nonconsanguineous parents from Brazil, was born at 38-wk gestation by Cesarean section for fetal distress. He was hypotonic and noted to have a left clubfoot at birth and was admitted to neonatal intensive care when episodes of upward rolling of the eyes were noted on the first day of life. MRI revealed extensive cortical malformation of the right hemisphere with PMG comprising the frontal, parietal, and temporal right lobes and the insula, and a small focus of signal abnormality in the periventricular white matter, compatible with recent ischemic injury. EEG revealed the presence of electroclinical seizures and his neurological examination at the time disclosed episodic dystonic posturing of the upper left extremity alternating with excessive movements of closing his left hand with thumb adduction. He was discharged on phenobarbital and levetiracetam. Upon neurological examination at 8 mo of age, he had a head circumference of 42.5 cm (−1.75 SD), very poor eye contact, global hypotonia with present reflexes, absent head support, absence of rolling and of object gripping, and presence of baby’s hand (Talon). WES revealed a de novo ATP1A3 variant, c.2771T > C (p.Leu924Pro), absent in both parents (Fig. 1 and SI Appendix, Fig. S1).

Case D: Extensive multifocal PMG (p.Gln851Arg). Case D, a male child of nonconsanguineous parents of European descent from the United States, was born at 41 wk by emergency Cesarean section; the pregnancy was complicated by fetal hydronephrosis. He developed electroclinical seizures and a neonatal encephalopathy requiring cooling and was discharged after 1 mo. MRI soon after birth showed multifocal PMG involving both cerebral hemispheres, right greater than left, with the right hemisphere smaller than the left (Fig. L4). At 4 mo of age he was admitted for congenital hip dysplasia surgery and noted to have frequent breath-holding spells that worsened after the surgery. His varied seizure phenotype included nystagmus, chin quivering, and hand shaking, which continue intermittently even through phenobarbital, levetiracetam, and oxcarbazepine combination therapy. At 15 and 30 mo of age, head circumference was 42.7 cm (−3.5 SD) and 44.2 cm (−3.3 SD), respectively. Physical examination at 18 mo demonstrated spastic hemiparesis involving primarily the left arm, but including the ipsilateral face and central hypotonia. Other symptoms included feeding difficulties and persistent constipation. WES revealed a de novo ATP1A3 variant, c.2552A > G (p.Gln851Arg), absent in the mother (father was not available for testing).

PMG-Associated ATP1A3 Mutations Are Predicted to Disrupt Function. ATP1A3 encodes the P-type Na⁺,K⁺-ATPase α3-subunit (NM_152296), an integral membrane protein that relies on ATP hydrolysis to transport ions (Na⁺ and K⁺) via 10 membrane-inserted helices (ITM1 to ITM10). This Na⁺,K⁺-ATPase is functionally supported by the β- and FYXD-subunits (7). ATP1A3 has several conserved domains across α-isomorph paralogs (α1 to α4) and orthologous genes (18), and is a highly constrained gene, with many fewer missense and LOF variants seen in the general population than predicted (gnomAD z-score of 6.33 and pLI score of 1.0, respectively). Taken together with ATP1A3’s known disease association with AHC, RDP, EIEE, and CAPOS (11), this suggests the identified variants in ATP1A3 are likely disease-causing (SI Appendix, Table S1).

PMG-associated ATP1A3 variants (p.Gln851Arg, p.Arg901Met, p.Leu924Pro, and c.2921+1G > A), and an AHC variant with PMG (p.Leu888Pro (17), occur at sites that are highly
conserved among the α-subunit paralogs (ATP1A1 to ATP1A4) and invariant in other vertebrate ATP1A3 orthologs (SI Appendix, Fig. S1B). In silico pathogenicity predictions for p.Gln851Arg, p.Arg901Met, p.Leu924Pro, and c.2921+1G > A suggest these variants are likely deleterious and disease-causing (SI Appendix, Table S1). Two amino acid substitutions reported here, Leu924Pro and Gln851Arg, have been previously associated with EIEE and AHC without MCD (13, 19), further supporting the wide spectrum of ATP1A3 phenotypes resulting from single amino acid variants (SI Appendix, Table S4). Leu924Pro pathogenicity has been demonstrated to affect ATPase trafficking to the plasma membrane, categorized functionally as LOF, leading to toxic effects in cells (13, 20). Together, the conservation of nucleotides, absence of variants in population databases, and previous LOF description of Leu924Pro (13, 20), suggest that these PMG ATP1A3 alleles are likely pathogenic.

PMG-associated missense ATP1A3 variants (p.Gln851Arg, p.Leu888Pro, p.Arg901Met, p.Leu924Pro) cluster within TM7 to TM8 segments (Fig. 1). The de novo ATP1A3 variant in Case A, NM_152296.4:c.2771G > T, p.Arg901Met (R901M), affects an arginine in the extracellular loop connecting TM7 and TM8 (Fig. 1C and SI Appendix, Fig. S1A), in the same segment as a previously described variant (p.Leu888Pro) occurring in an AHC individual with PMG (17). Additionally, these PMG-associated amino acids, Arg901 and Leu888, are in close proximity to Glu899, Gln900, and Gln905, which interact with the Na-K-ATPase β-subunit (21), suggesting these variants may affect the critical α-β interaction necessary for membrane localization (Fig. 1C). Case C possesses a de novo ATP1A3 variant, NM_152296.4:c.2771T > C, (p.Leu924Pro), altering an amino acid in the TM7 helix just upstream of the loop (Fig. 1C). Case D possesses a de novo ATP1A3 variant, NM_152296.4:c.2552A > G, p.Gln851Arg, altering an amino acid in TM7 (Fig. 1C). The clustering of these missense variants between TM7 and TM8 suggests a disruption of specific protein–protein interactions; on the other hand, the de novo ATP1A3 variant in Case B, NM_152296.4:c.2921+1G > A, abolishes the essential splice donor site of exon 21 (SI Appendix, Fig. S1), and similar ATP1A3 splice site variants are known to cause AHC (22). Therefore, the PMG-associated variants found in Case B and Case C likely represent the most significant lost Na+,K+-ATPase functionality, with the most severe PMG patient phenotypes likely reflecting the most severe LOF effects (i.e., Case B, splice donor variant likely resulting in haploinsufficiency), compared to other less-severe ATP1A3-associated conditions. Importantly, the four de novo-affected individuals did not possess any alternative variants in known MCD genes or related MCD pathways.

**ATP1A3 mRNA Expression in the Human Fetal Neocortex.** To investigate the transcriptional trajectory of ATP1A3 during human brain development underlying the prenatal phenotypes described in this study, we first contrasted the temporal expression patterns of ATP1A1 to -3 across brain regions (ATP1A4 does not maintain any significant level of brain expression), mining bulk neocortical transcriptome data from the Allen Brain Atlas (23), ranging from 12 wk postconception (wpc) to adulthood (40-y-old) (Fig. 2B). We find that prenatal expression level of ATP1A3 is higher than that of its paralogs (ATP1A1 and -2), and ATP1A3 persists postnatally (Fig. 2B). To gain further insights into the cellular biological mechanisms underlying the developmental cortical phenotypes described in this study, we characterized the patterns of ATP1A3 expression in the midgestational fetal human neocortex (19 to 21 wpe). This developmental age offers a uniquely informative window into corticogenesis as many and diverse biological processes—including neurogenesis, neuronal migration and differentiation, and cortical folding—occur at this age. At this stage, early-born ENs have settled and started to differentiate within the innermost strata of the cortical plate (CP; prospective layers 6a-5), while later-born ENs progressively migrate past their predecessors to occupy the most superficial aspect of the CP (prospective layers 4-2) (24). A large fraction of upper-layer ENs are still being generated in the germinal zones, while others—en route to the CP—are withheld within the SP, a transient developmental layer crucial for corticogenesis, where ENs are primed by thalamic input and form transient synaptic connections with one another and with resident SP neurons (prospective layer 6b) (25).

To investigate ATP1A3 expression in its tissue context, we performed RNA in situ hybridization (ISH) in coronal sections of fetal neocortex at 19 wpe from the perisylvian region, the area most commonly affected by PMG. ATP1A3 mRNA was virtually undetectable in the germinal zones where dividing neural stem cells reside (ventricular and subventricular zone, VZ and SVZ), but abundant in postmitotic neuronal layers, with highest expression in the inner CP and SP (Fig. 2C and D). Multiplexed fluorescent ISH analysis of ATP1A3 with markers of two populations of cortical NPCs, radial glia, and intermediate progenitor cells, confirmed ATP1A3 has low to zero expression in these dividing cells or their immediate progeny in either the VZ or the SVZ (Fig. 2C).

ATP1A3-associated diseases display a range of cerebral deficits throughout childhood development (11, 26, 27). We therefore temporally profiled cerebellar ATP1A3 expression from bulk RNA-seq data sampled from human embryonic week 12 to adult cerebellum (23). We show a differential enrichment of ATP1A3 expression to the prenatal period, compared to ATP1A1 and ATP1A2, yet ATP1A3 also persists postnatally (Fig. 2F). To better understand ATP1A3's developmental profile, we next performed ISH to evaluate ATP1A3 expression in fetal cerebellum tissue (19 wpe), demonstrating an exclusive enrichment of ATP1A3 within the Purkinje cell layer (Fig. 2E).

**Fetal Neocortex Single-Cell Analysis Demonstrates Robust ATP1A3 Enrichment to Resident SP Neurons and Rostral Neocortical ATP1A3 Enrichment.** Despite the high degree of compartmentalization of cell types into laminae, biological processes in the developmental neocortex are highly dynamic, and cellular compartments are heterogeneous in cell-type composition. scRNA-seq enables large-scale, simultaneous comparisons of gene expression across thousands of individual cells, thus bridging functional data from human genetics, immunohistochemistry, and cell-type–specific biology (28). Additionally, the limited spatial resolution of bulk RNA data from the Allen dataset does not allow cell-type identification within layers, which can result in miscategorized nonresident cells as they traverse layers (SI Appendix, Fig. S2G) (23). To this end, we performed Drop-seq on 125,942 individual cells from a 21-wpc human neocortex (Fig. 3A and SI Appendix, Fig. S24). Given the widespread pattern of PMG phenotypes from the perisylvian to frontal cortex localization described here, we sought to investigate ATP1A3 expression across presumptive neocortical areal domains. To build our single-cell expression dataset, we collected cells from 11 adjacent sections spanning the dorsomedial and rostrocaudal axes of the neocortex, validating and refining the positional identity of these serial samples using the graded expression of cortical patterning genes as benchmarks (Fig. 3A and S2 A–C) (29). Using unsupervised statistical methods (Materials and Methods), cells were partitioned into four transcriptionally distinct clusters, which corresponded to the major neuronal cell classes in the midgestational neocortex: ENs, INs, NPCs, and glia (Fig. 3B and C and SI Appendix, Fig. S2D). We excluded from this analysis all nonneural cell types as our scope of inquiry was specifically regarding ATP1A3 dysfunction in neurons, and nonneural expression of ATP1A3 is limited (Fig. 3B and SI Appendix, Fig. S2D). Within each of the main neuronal cell classes we could further resolve transcriptionally distinct cell subtypes and states, which we interpreted by the distinctive enrichment of marker genes in each group (SI Appendix, Fig. S2D). Among ENs, consistent with the...
developmental landscape of midgestational corticogenesis described above, we could identify newborn and migratory upper-layer ENs at different stages of differentiation (clusters EN-0 to -3), and postmigratory deep-layer ENs expressing marker genes of maturing layer 6-5, including markers of resident SP neurons (EN-4) (Fig. 3C). Across fetal cortical regions, ATP1A3 expression was observed in intermediate progenitor cell clusters, and more uniformly expressed in oligodendrocyte progenitor cells, INs, and ENs (Fig. 3C and SI Appendix, Fig. S2 D and E). Of these 18 cell types analyzed, the EN-4 cluster demonstrated the most significant ATP1A3 enrichment and the focus for detailed analysis.

The EN-4 cluster was composed of cells sourcing from all cortical regions, and was marked by enriched expression of SP-specific genes (CRYM and LPL) (Fig. 3C), as well as by the expression of genes associated with neuronal maturation (e.g., axonal genes NEFL/NEFM and synaptic genes SEMA3E) (Fig. 3H and SI Appendix, Fig. S2E). This is consistent with the notion that the earliest-born neurons (a subset of which will persist in L6b in the adult cortex) settle and mature early in the developing SP (30). Within this cluster, ATP1A3 appeared to be differentially expressed across the cortical region, with highest expression levels found in cells from frontal samples, intermediate levels in
Fig. 3. Single-cell ATP1A3 expression atlas in the developing human cerebral cortex. (A, Left) Schematic showing a 21-wpc forebrain and location of regions sampled (for anatomical detail, see SI Appendix, Fig. S2A). (Upper) Dorsolateral view; (Lower) sagittal view. (Right) Uniform Manifold Approximation and Projection (UMAP) of 125,943 single cells profiled by Drop-seq. Each dot represents a cell, color-coded by sample of origin. Key: blue-to-red: frontal-to-caudal. CGE, caudal ganglionic eminence; Hp, hippocampus; Ins, insula; MGE, medial ganglionic eminence; Occ, occipital; Orb, orbital; Par, parietal; PFC, prefrontal cortex; Str, striatum; Temp, temporal; V1, primary visual cortex. (B) UMAP plot of all profiled cells color-coded by level of ATP1A3 expression. (Inset) UMAP region highlighting highest ATP1A3 expression, which also maps onto ENs within the SP. (C, Left) Histogram showing relative expression of ATP1A3 across cell clusters (x axis; sample 1–11, ordered from caudal to rostral). Color scale codes for mean expression by group; size of the dots codes for percentage of cells expressing ATP1A3 in each group. (D, Upper) Violin-box plot showing differential expression of ATP1A3 across clusters split by areas of origin (x axis; sample 1–11, ordered from caudal to rostral). Color scale codes for mean expression by group; size of the dots codes for percentage of cells expressing ATP1A3 in each group. (D, Lower) Violin-box plot showing differential expression of ATP1A3 across clusters split by areas of origin (x axis; sample 1–11, ordered from caudal to rostral). Color scale codes for mean expression by group; size of the dots codes for percentage of cells expressing ATP1A3 in each group. (E) ATP1A3 expression across cells in the EN clusters ordered by pseudotime. Dots represent individual cells. Viols show probability density distributions; boxes show interquartile ranges; notches show confidence intervals around the median (horizontal lines). Outliers were trimmed. Mean expression of ATP1A3 was aggregated by region, then log10-transformed. Notice the highest expression of ATP1A3 in cells sampled from the frontal cortex, and lowest expression in cells sampled from caudal cortex. (Lower) Nissl-stained frontal coronal section of a 21-wpc forebrain highlighting anatomical position of SP. Source: Atlas of the Developing Human Brain, BrainSpan (www.brainspan.org) (64).
cells from medial samples, and lowest levels in cells sampled from more caudal cortical regions (Fig. 3C). In agreement with this, expression of ATP1A3 in the EN-4 cluster was found to be enriched in neurons coexpressing SP markers and frontal cortex markers, but much less so in neurons expressing caudal cortex markers (Fig. 3D and SI Appendix, Figs. S2 and S3).

Cortical development proceeds along a rostral-to-caudal maturation gradient, with asynchronous growth patterns accounting for the differential expansion of frontal and caudal cortical regions (31). This implies that, at any given time, neurons in frontal regions are on average more mature than their caudal counterparts. To investigate whether the observed ATP1A3 enrichment in the EN-4 cluster might reflect a differential maturation state of EN neurons along the rostrocaudal axis, we performed pseudotime analysis of ATP1A3 across all ENs (Fig. 3E). To do this, we used the Monocle3 algorithm, an established analytical tool allowing single-cell transcriptomes to be ordered and connected along a branched topology based on the differential expression of variable genes (32). By selecting an “origin” cell, this algorithm allows projection of a “pseudo-differentiation” trajectory onto the network topology (33). This analysis revealed a positive correlation between ATP1A3 expression and EN differentiation, and may suggest that ATP1A3 may be required during neuronal differentiation, as neurons transition to a mature state, most readily observed in the most distant SP neurons (Fig. 3B).

Finally, as the Na⁺,K⁺-ATPase is a heteromeric complex, we sought to identify potential isoform-specific and cell-type–specific vulnerabilities during corticogenesis; we compared the expression patterns of the ATP1A1 to -3 paralogs across cell clusters (SI Appendix, Fig. S3A). We found that expression of ATP1A2 is restricted to NPCs (and specifically to radial glia), and is virtually undetectable in neurons, in an almost mutually exclusive pattern to ATP1A3. ATP1A3 expression more closely mimicked ATP1A3 expression, albeit its expression in NPCs was higher, and partially overlapped ATP1A2 (SI Appendix, Fig. S3A). Accordingly, pairwise correlation analysis of the three isoforms across clusters revealed that expression of ATP1A1 and ATP1A3 correlates more strongly with the EN-4–SP cluster, while expression of ATP1A1 and ATP1A2 correlates more highly with NPC clusters (SI Appendix, Figs. S3 B and C). Across all cell-type clusters, pairwise correlation analysis indicated a positive correlation, though moderate, between ATP1A1 and ATP1A3, but weak-to-no correlation between ATP1A1 and ATP1B2, and between ATP1A2 and ATP1A3 (SI Appendix, Figs. S3 B and C). Interestingly, ATP1A3 also seems to have an exclusive enrichment to interneurons, not shared by ATP1A1. Taking these data together, this analysis suggests that single-cell variable expression of Na⁺,K⁺-ATPase isoforms could underlie compensation or vulnerability mechanisms for disease haploinsufficiency or differential penetrance within specific cell populations. Finally, we analyzed a previously published smaller human fetal scRNA-seq data (34) (~4,300 cells), which lends support to our findings that ATP1A3 is ubiquitously expressed across several cell types (SI Appendix, Fig. S9), with an EN enrichment.

**ATP1A3 Is Enriched in PV Interneurons and ENs in Infant Neocortex.** Given that ATP1A3 expression persists in the postnatal neocortex (Fig. 2B), and that several known ATP1A3 mutations show postnatal onset of symptoms, including epilepsy (11, 12), we profiled ATP1A3 expression in the infant neocortex. Using Drop-seq, we profiled 51,878 nuclei from an ~8-mo-old human cortex, sampled from prefrontal, temporal, occipital, and parietal lobes (Fig. 4A). Unsupervised hierarchical clustering sorted nuclear transciptomes into 3 major classes – ENs, INs, and glia – subdivided into 24 transcriptionally distinct cell classes (Fig. 4A and SI Appendix, Fig. S4). Within the EN clade, clusters stratified largely by layer and neuronal subtype, rather than cortical area, with several clusters enriched in gene markers of deep layers 5 and 6 (Fig. 4B; see SI Appendix, Fig. S4 for full cladding). Within the IN clade, cells stratified by developmental origin (caudal or medial ganglionic eminence) and major “cardinal types,” according to previous studies (35). In the infant neocortex, similar to the fetal neocortex, ATP1A3 was enriched in neurons compared to glia, but unlike in the fetal dataset, ATP1A3 was more highly expressed in INs than in ENs (Fig. 4B and SI Appendix, Fig. S5).

Among neuronal clusters, and across cortical regions, ATP1A3 expression was fairly variable, with the lowest expression in V1/occipital cortex cells, and wide range of expression levels within deep and upper layer EN clusters (Fig. 4B). However, no specific enrichment of ATP1A3 expression was found in L6b ENs (i.e., the postnatal remnant of the fetal SP layer), suggesting that fetal enrichment of ATP1A3 in the SP is developmentally transient, with maturation and migration of cells continuing to populate the upper layers. Of note, the lack of specific enrichment in postnatal SP may also reflect the fact that, unlike the fetal dataset, the postnatal dataset was built from nuclear RNA rather than whole-cell RNA, so that some transcripts may be underdetected. Across IN clusters, expression of ATP1A3 was highest in cells sampled from the frontal lobe (prefrontal BA10), and more strikingly so within PV+ INs (Fig. 4B and SI Appendix, Fig. S4). This differential expression pattern raised the hypothesis that ATP1A3 may be required dynamically across neuronal types, cell-biological processes, and across developmental ages. To gain insights into the nature of the cell-biological signature associated with ATP1A3 expression, we compared the top 100 genes correlating with ATP1A3 in EN and IN clusters. Interestingly, we found that 50 of 100 ATP1A3-correlating genes were shared across cell types, suggesting that ATP1A3 partakes in similar transcriptional programs, and thus potentially similar function, in different neuronal types. In both ENs and INs, among the top genes correlating with ATP1A3 was FXD/Y6, the FXD/Y6 subunit of the ATPase complex (Fig. 4D). We did find, however, that the β-subunit ATP1B1 (β1) correlated with ATP1A3 only in ENs, and ATP1B2 (β2) in INs, suggesting that, at least in part, ATP1A3 may form cell-type–specific heteromeric ATPase complexes in different neuronal classes (Fig. 4D and S3 A and B). Gene ontology (GO) enrichment analysis of the top 100 genes most strongly correlated with ATP1A3 in both ENs and INs revealed significant overrepresentation of gene products associated with TM tonic transport and the somatodendritic compartment, including dendrites and synapses (Fig. 4D and SI Appendix, Table S2). This is consistent with the established role of ATP1A3 in synaptic transmission and membrane potential and suggests that ATP1A3 may partake in a shared transcriptional network for which ATP1A3 variants may perturb aspects of neuronal physiology central to many cell types at different stages of their maturation process.

Given the association of ATP1A3 pathological variants with human disease phenotypes affecting higher-order cognitive functions with no clear counterpart in animal models, it was of interest to investigate the degree to which ATP1A3 enrichment in INs may be conserved among humans, nonhuman primates, and rodents. We took advantage of a recently published scRNA-seq dataset profiling gene expression in homologous IN types in humans, nonhuman primates, and rodents. We found that relative ATP1A3 expression levels across IN types, both in the neocortex and in the striatum, are largely conserved across species, with shared highest ATP1A3 expression in the fast-spiking PV+ INs (SI Appendix, Fig. S6). This suggests that the observed differential expression of ATP1A3 across neuronal types is genetically encoded and evolutionarily conserved.

**Discussion**

Mutations in ATP1A3, the gene encoding the Na⁺,K⁺-ATPase β3 catalytic subunit, have been associated with a wide spectrum of neurological diseases affecting childhood development, ranging in onset age from early infancy to late adolescence into adulthood.
Here, we describe \textit{ATP1A3} pathogenic variants in individuals presenting with PMG, a severe prenatal malformation of the cerebral cortex characterized by abnormal gyrification and laminar organization. These findings extend the known phenotypic range of \textit{ATP1A3}-related diseases and provide new insights into the molecular pathology of PMG and PMG-associated developmental channelopathies. Furthermore, by compiling a single-cell atlas of \textit{ATP1A3} expression in the fetal and early postnatal neocortex, we revealed cell-type–specific \textit{ATP1A3} expression in the fetal and infant human brain, establishing a spatiotemporal roadmap for future genotype–phenotype associations studies, and studies of \textit{ATP1A3} function in the SP and cortical interneurons.

\textit{ATP1A3}-associated conditions comprise a broad spectrum of overlapping phenotypes, including the blending of encephalopathies with more paroxysmal phenotypes, with a complex genotype–phenotype correlation. Further confounding genotype-to-phenotype studies, identical amino acid substitutions can impart variable presentations in humans (SI Appendix, Table S4); for example, Leu924Pro and Gln851Arg reported here have been previously associated with EIEE and AHC without MCDs (13, 19), but with postnatal microcephaly (Leu924Pro), suggesting pathogenic severity and susceptibility may differ between individuals. Differential penetrance between individuals may reflect α3 ATPase expression levels, competition between disease and
wild-type α3-subunits for utilization within Na,K-ATPases (13), and as described in this study, variable expression of α3 (and coexpression with other α- and β-subunits) within different cell populations (Fig. 4D and SI Appendix, Figs. S3 and S5). Individuals with heterozygous ATP1A3 variants presented here with PMG also exhibited a range of phenotypic severity, from unilaterial to extensive bilateral PMG, variable degrees of developmental delay, postnatal microcephaly, ataxia-like phenotypes, and EIEE. ATP1A3's wide phenotypic spectrum is similar to known glutamate and sodium channel “developmental channellopathies,” in which a subset of cases present with EIEE and developmental delay, both with and without MCDs and postnatal microcephaly (1). In a future opportunity to examine cortical tissue of PMG associated with ATP1A3 variants, it would be useful to document cortical architecture, including whether gaps occur in the pia mater and glia limits. This anatomical defect permits continuity between the molecular zones of adjacent microgyri for synaptic excitatory short-circuitry, leading to epilepsy, as with other genetic mutations in PMG (16).

Functional analysis of ATP1A3 variants associated with postnatal diseases are largely described as LOF, including increased endoplasmic reticulum retention, reduced α3 expression, and disrupted cell morphology for L924P associated with EIEE (13, 28). Additional LOF mechanisms described associated ATP1A3 disease-causing triggers include changes in Na+ affinity, intracellular conformational changes, voltage-dependence shifts, and disruption of protein expression, among others (13, 37, 38). Since the Na,K-ATPase exports Na+, and elevated Na+ can be deleterious to cellular processes (39, 40) and is a known PMG-causing pathway (1, 4, 41), α3 variants that result in increased intracellular Na+ concentration (e.g., a shift in Na+ affinity or reduced ATPase in plasma membrane) could share this known PMG pathophysiology (1, 4, 41). This would suggest the toxicity resulting from inability to maintain sufficient levels of Na,K-ATPase, as with the deleterious splice variant likely causing haploinsufficiency (Case B), may also be correlated with patient phenotype severity (Individual B).

While some ATP1A1 variants have been described to increase inward leak of Na+ (42, 43), similar pathogenic gain-of-function effects have not been described in ATP1A3-related diseases. L924P may also activate a proapoptotic pathway (20), thus PMG patterns of injury in ATP1A3 variants are not only reflective of toxic downstream mechanisms that underlie postnatal, ATP1A3-related disorders causing neurodegeneration or cell death, including postnatal microcephaly (44), regional neuronal loss (45), and cerebellar hypoplasia (26, 27). Moreover, two of the PMG-causing ATP1A3 variants we describe are located in the α3 TM7/TM8 extracellular segment that interacts with the β-subunit (21) and Leu924Pro demonstrates β-subunit–associated LOF membrane localization properties (13, 20), suggesting the final formation of α-β complex could potentially underlie the observed pathophysiology. The β-subunit supports localization of the α-subunit to the plasma membrane and modulates affinity of Na+ and K+ for the ATPase (46), including a differential β1 vs. β2 effect on α-subunit ATPase pump activity (47). This suggests the observed cell-type–specific α-β combinations, including α3-β1 and α3-β2 enrichments in ENs and INs, respectively, could confer differential cell-type–specific pathology. Finally, α- and β-subunits can trigger intracellular signaling pathways associated with MCDs, including MAPK and AKT signaling via phosphoinositide 3-kinase (PI3K) and EGFR pathways, resulting in changes in cell polarity, growth, motility, and gene expression (48).

During embryonic brain development, bioelectric cellular properties are required for the propagation of key organizing signals, directly implicating ion channels and pumps in a variety of morphogenetic processes (15). For example, in mice and ferrets, experimental modulations in membrane potential and Na+-dependent excitation have been linked to neuronal fate specification and directed migration (4–6, 49). Early electrical activity patterns in the SP accompany the dynamic formation of transient synaptic networks between resident SP neurons and migratory ENs and INs en route to the CP, ascending radial glia processes, and ingressing thalamic fibers (25). Thus, the SP acts to spatiotemporally integrate signals from many cellular sources. Our finding that 1) ATP1A3-expressing cells are located in the developing SP (ISH) (Fig. 3D), and 2) coexpress SP-specific marker genes (Drop-seq) (Fig. 4 and SI Appendix, Fig. S2) is therefore consistent with a role for ATP1A3 in supporting electric activity within this compartment. This is also consistent with the notion that human SP neurons possess large Na+ currents with prolonged depolarized states (50), and therefore require a stout mechanism to recover Na+ and K+ electrochemical gradients following excitation, a known function of the α3 ATPase in postnatal neurons (9, 10, 51). In addition, the finding that ATP1A3-expressing cells are also found in the inner aspect of the CP (ISH) (Fig. 2C), express L6-S EN markers (Drop-seq) (Fig. 3C), together with the rostral-to-caudal gradient of ATP1A3 expression and positive correlation with neuronal pseudode (Monocle) (Fig. 3F), all are consistent with a role of ATP1A3 in the maturation of early-born deep-layer ENs.

The observed differential prenatal expression of Na+, K+, ATPase isoforms (ATP1A1 to -3) parallels the enrichment pattern reported for specific glutamate and sodium channel subtypes implicated in developmental channelopathies (1). These glutamate and sodium channel diseases result from mutations in developmental ion channel subtypes, specific channel subtypes that are utilized for developmental electrical functions (SCN3A, GRIN2B) (1), and contribute to neurotransmitters cellular responses (52), as opposed to others that support postnatal electrical functions (SCN1A, GRIN2A) (1). Similar to SCN3A and GRIN2B, the α3 isoform may have a similar specialized function in fetal brain neurons (compared to α1 isoforms), including interneurons. For example, Na+ affinity is lower in α3 than α1 isoforms (39, 53), enabling α3-containing ATPase pumps to respond to larger Na+ influxes. Lower Na+ affinity provides a wider range to buffer aberrant Na+ flux, therefore could provide an evolutionarily advantageous neuroprotective effect in cells during development. In addition to neurophysiological differences, known developmental channelopathy genes also maintain a robust cell-type and subtype expression profiles as ATP1A3; for example, SCN4A and GRIN2B are enriched in INs and some progenitors (54), before transitioning to mostly GRIN2A-containing glutamate receptors and SCN1A in the postnatal cortex (1, 54). Moreover, the observed scRNA-seq ATP1A2 enrichment in NPCs could offer a pathological basis for ATP1A2 variants previously associated with fetal or early life demise, including microcephaly (55, 56).

In the postnatal cortex, the observed ATP1A3 enrichment in interneurons likely supports physiological processes for maintaining inhibitory tone of cortical circuits (48), a proposed mechanism underling EIEE, or perhaps AHC. We were surprised to find that even after 90 million years of divergence, the human and rodent postnatal cortex possess similar single-cell distribution of ATP1A3, most notably in PV+ INs, underscoring the utility of mice as a model for studying excitatory/inhibitory balance (57), especially in the context of PV IN loss relating to postnatal brain disorders (58). On the other hand, while ATP1A3 IN expression appears to be conserved, species-specific differences in circuits and connectivity, and how interneurons shape gene expression differentially, could offer pathological differences between humans and mice, including early connections between SP and somatostatin/PV+ cells (25, 30). For example, the observed PV IN enrichment of ATP1A3 in the prefrontal cortex could contribute an early susceptibility for ATP1A3 variant-associated childhood onset schizophrenia (12, 59). Intriguingly, the highest expressing ATP1A3 cell types in the pre- and postnatal cortex (SP and PV+ neurons), and Purkinje cells of the cerebellum, are all characterized by their robust firing properties (50, 60). Within these cell
types with more dynamic physiological needs, the low-affinity α3 would help maintain ionic gradients following excitation and suggests neurons with higher neurophysiological demands would be most susceptible to Na⁺,K⁺-ATPase dysfunction. In addition to differences between EN and IN Na⁺,K⁺-ATPase activity (61), differential Na⁺,K⁺-ATPase activity levels exist within mouse cortical pyramidal neurons in the postnatal brain (62). Taken together, the suspected LOF PMG-associated ATPA3 variants likely result in combined disruption of biosynthesis and pump dysfunction within vulnerable cell populations, with the most severe phenotype variants reflecting increased cytotoxicity capable of disrupting early neocortical developmental processes.

Materials and Methods

Human Subjects and Samples. Individuals presented herein were identified and evaluated in a clinical setting, and biological samples collected after obtaining written informed clinical and research consent. Human subject research was conducted according to protocols approved by the institutional review boards of Boston Children’s Hospital, Beth Israel Deaconess Medical Center, and the Mayo Clinic.

Human Genetics Sequencing and Analysis. For Cases A and B, WES and data processing were performed by the Genomics Platform at the Broad Institute of MIT and Harvard. Libraries from DNA samples (>250 ng of DNA, >2 ng/μL) the Human Core Exome Kit from Twist Biosciences was used to capture target regions (38–58 Mb target), and sequencing was performed on the illumina NovaSeq. 6000 (150-bp paired reads) to cover >96% of targets at 20x and a mean target coverage of >100x. Sample identity quality-assurance checks were performed on each sample. See SI Appendix for additional information.

Phenotypic Assessment. All affected individuals and clinical data were examined by neurologists and geneticists and radiologists, and PMG was diagnosed using criteria described previously (1, 16). The SI Appendix summarizes their phenotypes and detailed clinical and radiographic evaluations.

Human Brain Tissue Preparation and mRNA ISH. Human brain tissue preparation and mRNA ISH were performed as previously described by Smith et al. (4). Briefly, following fixation (4% PFA) and cryoprotection (30% sucrose), brains were frozen using isopentane on dry ice. Samples were sectioned at 20- to 30-μm thickness (Leica Cryostat), mounted immediately onto warm charged SuperFrost Plus slides (Fisher), and stored at −80 °C. We followed the manufacturer’s standard protocol for multiplex fluorescent ISH (Multiplex v2 kit, Advanced Cell Diagnostics). See SI Appendix, Supplemental Materials and Methods for more details.

Bulk Human Cortex Gene-Expression Analysis. The Allen Human Brain Atlas (ABA) published a rich dataset of cortical genetic expression across cortical brain regions from age 8 wpc to adult ages (23). BrainSpan data analysis of ATPA13 (chr1:116,915,289–116,952,883, GRCh37/hg19), ATPA12 (chr1:160,085,548–160,113,381, GRCh37/hg19), and ATPA13 (chr19:42,470,733–42,498,384, GRCh37/hg19) was performed. ATPA14 is not expressed in the CNS and was not included as the read counts were near zero. See SI Appendix, Supplemental Materials and Methods for more details.

Drop-Seq Isolation and Sequencing. Single-nucleus and single-cell suspensions were processed for Drop-seq as described in detail (5, 6), with modifications detailed by Krienen et al. (36) (see SI Appendix for additional details). Single-cell cDNA libraries were processed and sequenced using illumina short-read sequencing on a Nova-seq platform at the Broad Institute. Libraries were sequenced at an average depth of >8 reads per unique molecular identifier (UMI). Sequencing reads were aligned to the hg19 human reference genome. For the fetal dataset, only reads mapping to exons were used; for the postnatal dataset, reads mapping to both exons and introns were used. Software and core computational analysis for alignment and downstream processing of Drop-seq sequencing reads are freely available at https://github.com/broadinstitute.Drop-seq. Cell selection was performed as described previously (63). See SI Appendix, Supplemental Materials and Methods for independent analysis component, clustering, and statistical analysis details.

Graphics. Graphics were prepared in Adobe Illustrator and using BioRender software.

Data Availability. Sequencing data have been deposited in dbGap, https://www.ncbi.nlm.nih.gov/gap/ (accession no. phs001272.v1.p1) (65). Supplemental data tables are available at https://figshare.com/s/56b48891e4817f9e123. All other data are included in the main text and SI Appendix.

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