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Interneuron, Interrupted: Molecular Pathogenesis of ARX Mutations and X-linked Infantile Spasms

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Abstract

X-linked Infantile Spasms Syndrome (ISSX) is a catastrophic epilepsy of early childhood with intractable seizures, intellectual disability, and poor prognosis. A spectrum of mutations in the Aristaless-Related Homeobox gene (*ARX*) has been linked to ISSX, and downstream targets of this interneuron-expressed transcription factor are being defined. Recent advances combining *in vitro* and *in vivo* methods have unveiled complex interactions between Arx and its binding partners and their effects on cell migration and maturation that can help explain the diversity of *ARX* phenotypes. New mutant mouse models of Arx-induced pathology, including a recent human triplet-repeat expansion mutation with a phenotype of infantile spasms and electrographic seizures, provide valuable tools for exploring the pathophysiology of Arx and substrates for testing novel therapies.

Introduction

X-linked Infantile Spasms Syndrome (ISSX; OMIM ID: 308350) is a rare and catastrophic epilepsy syndrome of childhood that consists of early-onset myoclonic spasms, irregular high voltage EEG waves termed hypsarrhythmia, followed by intractable seizures and intellectual disability (ID) associated with autistic features. ISSX is one of over a dozen infantile spasms syndromes for which single genes have been discovered in the last decade, triggering a molecular reclassification of this major category of pediatric epilepsy [1*]. While the clinical spasms usually subside by age 5, epilepsy and ID persist through adulthood. Although a brief course of adrenocorticotropic hormone (ACTH) therapy can control spasms in the short term, there are concerns about long-lasting side effects and its

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overall efficacy [2, 3, 4]. In addition, no current therapies have been shown to prevent epilepsy or improve the intellectual outcome of patients with ISSX [5, 6, 7, 8, 9]. Therefore, understanding the mechanisms underlying ISSX and developing novel therapies remain critical scientific and clinical priorities.

The last decade has witnessed critical advancements in our understanding of the pathophysiology of the syndrome, beginning with the finding that expansions in the first and second poly-alanine tracts (pA1 and pA2) in the transcription factor gene **Aristaless-Related Homeobox** (***ARX***) (GenBank ID: NG_008281.1) cause ISSX [10, 11, 12, 13]. *ARX* mutations cause a spectrum of clinical disorders, from X-linked Lissencephaly and Ambiguous Genitalia (XLAG; OMIM ID: 300004) to ISSX and non-syndromic mental retardation (OMIM ID: 300419) [14]. In the CNS, *ARX* is expressed in subpallial proliferative zones and in developing and adult interneurons, where, under the influence of DLX1/2 and a downstream network of transcription factors, it plays a pivotal role in migration and differentiation [15, 16, 17, 18, 19, 20, 21].

The first mouse model to be generated, the constitutive *Arx* knockout mouse (Arx^{KO}), displays XLAG-like characteristics with a small brain and severe interneuron migration deficit. Arx^{KO} mice die soon after birth, preventing the study of the role of Arx in postnatal neurodevelopment [22] (Table 1). To overcome this limitation, several groups have developed postnatally viable rodent models of *Arx* mutations featuring full longevity. Also, the past two years have seen significant progress in exploring the molecular pathology of *Arx* mutations and their impact on gene expression during development.

Mutations in ARX domains lead to distinct phenotypes

ARX has several conserved domains: the octapeptide domain, 3 nuclear localization sequences (NLS1-3), 4 poly-alanine tracts (pA1-4), the DNA-binding homeodomain (HD) and the *Aristaless* domain [11,15] (Fig. 1). Truncations and HD mutations commonly cause severe phenotypes, whereas missense mutations and in-frame expansions of the first two pAs associate with the more neurologically restricted *ARX* phenotypes, such as ISSX [14]. Despite this apparently consistent genotype-phenotype relationship, precise details on the complex molecular pathology of different *ARX* mutations are poorly understood. In recent years, several attempts to reproduce the most common *ARX* mutations and investigate their downstream effects have appeared and are reviewed here.

The transfer of Arx into the nucleus is mediated by **importins**, which transiently bind to the NLS and release Arx inside the nucleus by a RanGTP-dependent mechanism [23,24]. In an *in vitro* trafficking study, Arx proteins with naturally occurring NLS mutations and overexpressed in HEK293 cells accumulated next to the nuclear membrane and co-localized with the importin, IPO3. In contrast, WT Arx diffusely stained throughout the nucleus and did not co-localize with IPO3, suggesting that IPO3 normally rapidly dissociates from Arx once inside the nucleus. Interestingly, binding of NLS mutated Arx to IPO3 was indistinguishable from WT Arx. However, mutant Arx proteins did not dissociate from IPO3 once inside the nucleus, suggesting that the principal defect is the inability of mutant Arx to uncouple from IPO3 [25*].

Arx directly represses the transcription of *Lmo1, Ebf3*, and *Shox2* [26,27**] (Fig. 2). To investigate how missense HD mutations may affect this repression, Shoudbridge *et al.* (2011) measured transcript levels of *Lmo1* and *Shox2* in HEK293T cells transiently overexpressing either WT Arx, NLS, or HD mutants. Electrophoretic mobility shift assays (EMSA) and ChIP-qPCR data suggest that HD mutations lead to de-repression of Arx targets by loss of DNA binding. NLS mutations also result in transcriptional de-repression,

which could be explained by the formation of peri-nuclear aggregates. Conversely, HD mutants would not be expected to mislocalize or form aggregates, yet some HD mutations (i.e. L343Q, P535R, and R358S) display double the rate of protein mislocalization relative to WT-Arx. Although below the 5-7 fold rate of abnormal protein localization observed in NLS mutants, this result raises the possibility that some HD mutations partially disrupt nuclear translocation [28*].

A 24-bp duplication in pA2 and a 7-alanine expansion in pA1 are both common causes of ISSX, although other mutations have been reported [10,11,13]. The connection between relatively small in-frame expansions in a poly-alanine tract and the cellular pathogenesis of infantile spasms, seizures, and intellectual disability is unclear. One hypothesis, that expansions of Arx lead to misfolding and aggregation, has gained traction after the finding that overexpression of Arx with expanded pA1 and pA2 *in vitro* caused Arx mislocalization and increased cell death [23,29]. Recently, heterologous overexpression of expanded Arx proteins in HEK293 cells produced similar results [30]. New mutations in pA1 and pA2 found in patients were introduced into HEK293T cells. The degree of protein mislocalization correlated with the length of the pA expansion and phenotypic severity. Protein mislocalization was also observed in Arx proteins containing point mutations in the HD [28*]. Although nuclear translocation defects and protein misfolding can result in mislocalization, it is possible that the degree of mislocalization and aggregation observed in heterologous overexpression systems may not be the same in developing neurons. In fact, the recently reported pA1 expansion mouse model does not show increased cell death, and protein mislocalization is nonuniform, appearing predominantly in a subset of interneurons in the somatosensory cortex [31**]. Therefore, protein mislocalization may only partially explain Arx pA1 expansion cellular pathology; and other defects in regulation of gene expression may be contributing factors. *In vivo* data from electroporation of Arx^{E} (8-alanine expansion in pA1) in $Arx^{-/Y}$ embryos support this notion. While WT-Arx rescued radial and tangential migration, Arx^{E} partially rescued radial, but not tangential, migration. This was accompanied by a selective transcriptional de-repression of Arx direct targets, raising the possibility that radial and tangential migration modes require expression of distinct gene subsets [32**]. These results disagree with *in vitro* data where increasing the length of pA1 and pA2 resulted in increased transcriptional repression as measured by a reporter vector [33].

Co-factors can refine the activity of transcription factors with cell-type specificity. In the case of Arx, the co-repressor Tle1 displayed diminished binding to Arx^{E} *in vitro*. It is possible that differential expression of Tle1 and other unknown Arx co-factors across the forebrain may partially explain the different effects of Arx^{E} on radial and tangential migration and the broad spectrum of *ARX* phenotypes [32**,33]. Overall, these studies paint a complex picture where protein mislocalization and selective gene deregulation both contribute to the molecular pathogenesis of Arx pA expansions.

Transcriptional control of Arx targets

Mutations that impair DNA binding, nuclear translocation, or interactions with co-repressors are likely to differentially impact the expression of Arx targets, which may partially explain the broad spectrum of *ARX*-related phenotypes. In fact, relatively subtle single nucleotide homeodomain mutations display significant differences in transcriptional repression [34]. In *C. elegans*, deletion of the *Arx* orthologue, *alr-1*, results in a highly variable touch-insensitive phenotype, suggesting a role for *alr-1* in maintaining a high expression level of its targets by minimizing stochastic variation in their transcription [35*]. Although it is difficult to translate these results to mammalian brain, they may contribute to the clinical

variability in humans with *ARX* mutation, notwithstanding environmental and genomic individual differences.

Given the dimensions and severity of human *ARX*-related disease, the identification of novel ARX targets and elucidation of their individual roles in neurodevelopment are high priorities. Recent microarray and qPCR efforts have led to the discovery of 35-84 genes potentially regulated by Arx. Three of these genes have been confirmed as direct targets: *Lmo1, Shox2* and *Ebf3*. In addition, Arx modifies the brain expression of *Lmo3, Lmo4, Cxcr4, Cxcr7*, and *Lhx7*, among others [26,27**]. Cxcr4 and Cxcr7 are receptors that bind stromal cell-derived factor α (SDF-α) and play distinct roles in interneuron migration [36, 37, 38, 39, 40, 41]. The transcription factor gene *Ebf3* is strongly repressed by Arx and its ectopic expression in *Arx* null mutants halts the normal migration of interneurons, indicating that its gene targets are key regulators of cortical development. In contrast, ectopic expression of *Lmo1*, another transcription factor gene normally repressed by Arx, did not result in detectable migration deficits [27**]. Dozens of potential novel downstream targets have recently been identified using a high-throughput method to detect Arx binding to promoter regions, enriching the already complex pool of genes controlling interneuron development that may be regulated by Arx [42] (Fig. 2).

While *in vitro* methods have permitted a rapid growth in our understanding of Arx-driven molecular pathology and transcriptional defects, the creation of rodent models engineered with *Arx* mutations remains a necessary step for investigating how endogenously expressed mutant Arx proteins alter specific steps in interneuron development leading to the neural circuit deficits and clinical features of ISSX.

Engineered *Arx* poly-A expansions in mice reproduce human *ARX*/ISSX phenotypes

While the first constitutive *Arx* knockout mouse model highlighted the importance of Arx in development, the null mutant dies shortly after birth, limiting its usefulness for studying the role of Arx in adulthood [22]. To test the hypothesis that Arx deficiency in developing interneurons is sufficient to replicate features of ISSX, Marsh *et al.* (2009) created a viable conditional knockout model of *Arx* by deleting the gene only in cells within the medial ganglionic eminences. The conditional *Arx* knockout (***Arx*$^{-/Y}$; *Dlx5/6*^{CIG}**) displays spontaneous seizures by day P14, abnormal EEG patterns, and loss of calbindin immunoreactive neocortical interneurons. The presence of infantile spasms was not noted in this model. Interestingly, heterozygous females also showed spontaneous seizures, but not as frequently as male hemizygotes [43*]. Although there are no known human null mutations reported, the *Arx*$^{-/Y}$; *Dlx5/6*^{CIG} mouse represents a unique opportunity to study the role of Arx in interneuron development in brain tissue with an otherwise wild-type background, which may provide additional clues regarding interneuron specific mechanisms underlying ISSX (Table 1).

Recently, two mouse models engineered with expansions in pA1 which reproduce the human 7-alanine expansion have been independently developed [31,44**]. The model published by Kitamura and colleagues, ***Arx*$^{(GCG)7/Y}$**, survived for approximately 3 months, allowing the detection of spontaneous seizures, learning impairments, loss of cholinergic and GABAergic interneurons, and interneuron migration deficits in the developing brain. In their study, two other models with homeobox domain mutations, P355L and P355R, were also made for comparison. The ***Arx*^{P355L}** (or *Arx*^{PL}) line had a similar, but more subtle phenotype compared to *Arx*$^{(GCG)7/Y}$. These features stood in striking contrast to the ***Arx*^{P355R}** (or *Arx*^{PR}) model whose mutation lies in the same position within the gene, yet causes perinatal death, gross brain malformations, and severe defects in interneuron

migration similar to the *Arx*$^{-/Y}$ mouse and human XLAG [44**]. It has been shown that an expanded Arx protein at the pA1 results in misregulation of Arx transcriptional targets [23]. Therefore, it is possible that the P355L and the (GCG)7 mutations result in a similar defect in transcriptional regulation (Table 1, Fig.1).

The model developed in our laboratory has a similar 7-alanine (GCG)7 expansion mutation, but with a notable difference. Unlike the human *ARX* gene, where pA1 has 16 alanine codons (10 of which are coded by GCG), the pA1 in the mouse *Arx* contains 15 alanine codons (4 of which are coded by GCG). To achieve the same 23-alanine long pA1 tract mutation seen in humans, an expanded poly-alanine mutation was inserted in the pA1 of the mouse orthologue such that it contained a total of 23 alanine residues. This humanized mouse model of ARX pA1 expansion, denominated ***Arx*$^{(GCG)10+7}$**, has a normal life span, and displays spontaneous seizures and frequent interictal spikes in the EEG. In addition, *Arx*$^{(GCG)10+7}$ mutant pups display a transient pattern of dyskinetic motor spasms during the first two weeks of life resembling motor spasms observed in humans with ISSX [31**]. Notably, hypsarrhythmia, an EEG abnormality commonly seen in association with human infantile spasms, was not observed in *Arx*$^{(GCG)10+7}$ mutants studied at 2 weeks postnatal, although mice were not examined for this EEG phenotype at earlier postnatal stages when spasms were present due to technical challenges. Hypsarrhythmia has not been observed in all ISSX cases with a known orthologous ARX triplet repeat expansion [45]. *Arx*$^{(GCG)10+7}$ mutant mice do display impaired learning ability and social interactions, which parallel the human ID syndrome (Table 1).

At the neuropathological level, the *Arx*$^{(GCG)10+7}$ mutation causes a partial loss of neocortical calbindin- and NPY-positive GABAergic interneurons and loss of striatal NPY-, calbindin-, and ChAT-positive neurons. Parvalbumin- and calretinin-positive neurons are largely spared. This pattern of interneuronal loss suggests that specific regional patterns of synaptic dysinhibition may generate the complex phenotype of epilepsy, motor spasms, and cognitive impairment. Intracellularly, the molecular lesion also displays complexity, since cytoplasmic inclusions in the *Arx* mutant are seen in somatosensory cortex neurons but not observed elsewhere in the forebrain, suggesting that protein mislocalization may be cell-type specific rather than playing a uniform role in in the development of the *Arx*$^{(GCG)10+7}$ phenotype [31**]. The viability of the *Arx*$^{(GCG)10+7}$ mouse model containing the human-like 23-alanine expansion in pA1 with a phenotype closely resembling human ISSX makes it a valuable tool for studying the pathophysiology and potential future treatment strategies of *ARX* diseases *in vivo*.

Conclusion

The recent studies on how *Arx* mutations alter its function, the identification of novel transcriptional targets, and the creation of clinically relevant models of *ARX*/ISSX represent a significant advance toward understanding the pathogenesis of human ARX-mediated disease. Alterations in gene expression patterns driven by Arx mutations have highlighted genes whose precise roles in neurodevelopment and the rise of ISSX and other Arx-related disorders have yet to be described. While *in vitro* studies continue to provide a framework for exploring the molecular pathology and cellular consequences of various Arx mutations, the recent generation of several models of Arx deficiency based on naturally occurring human mutations has provided valuable tools to validate downstream gene changes in the context of the developing brain, and an opportunity to link these with human neurological phenotypes. These models are also likely to provide useful substrates for investigating novel therapies for Arx-related diseases, for which only limited treatment options exist.

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Highlights

- ARX is a transcription factor deeply involved in interneuron development
- *ARX* mutations can cause epilepsy, infantile spasms, and intellectual disability
- Downstream consequences of *Arx* mutations reveal complex molecular pathogenesis
- Mouse models of human *ARX* mutations are valuable tools to study role of *Arx in vivo*
- New mouse models provide a substrate for the investigation of novel therapies

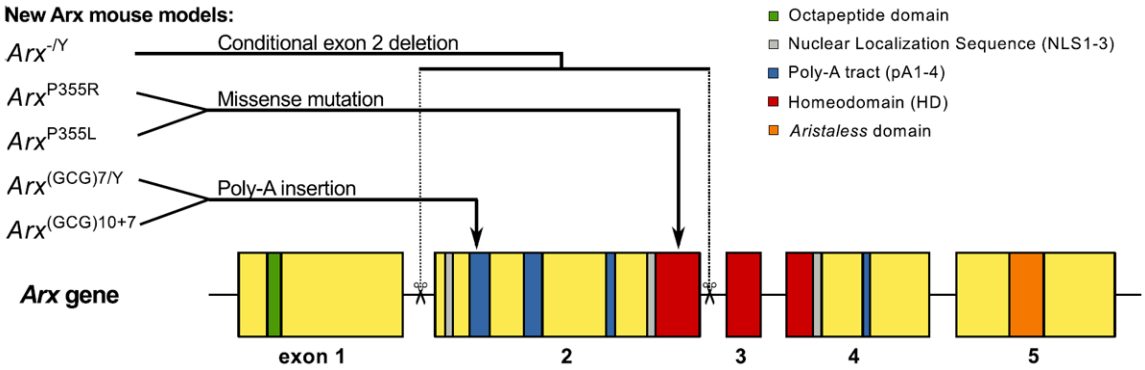

Figure 1.

Diagram of Arx gene and its major conserved functional domains. These domains include the Octapeptide domain, 3 Nuclear Localization Sequences (NLS1-3), 4 poly-alanine tracts (pA1-4), the DNA binding Homeodomain (HD), and the *Aristaless* domain. The general location of the mutations of current experimental Arx mouse models is shown.

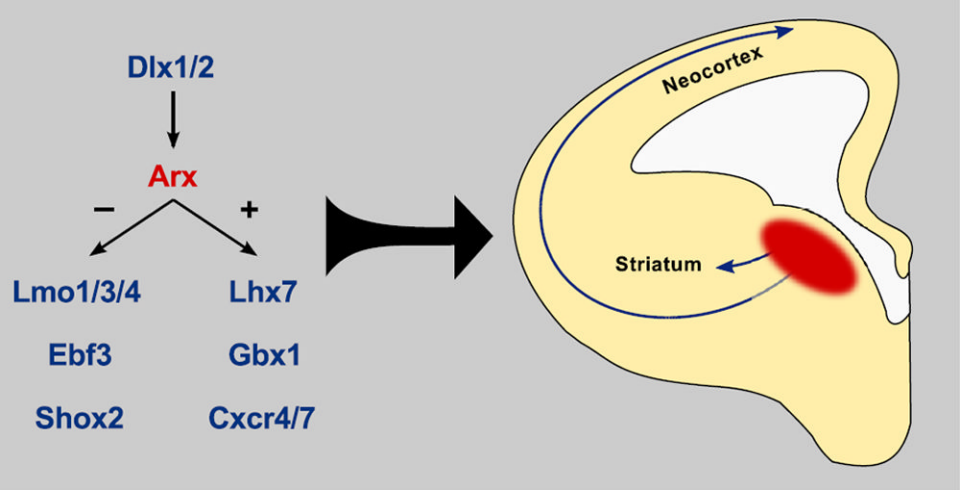

Figure 2.

Arx regulates transcription of genes involved in interneuron development. The transcription factors Dlx1/2 upregulate Arx expression. Arx itself acts as both a transcriptional suppressor and an activator. Arx regulates transcription of several genes, including other transcription factors such as Lhx7, Gbx1, Ebf3, Lmo1/3/4, and Shox2, each of which play distinct roles in interneuron proliferation, migration, and differentiation. Other targets, such as Cxcr4, encode chemokine receptors directly involved in regulation of interneuron migration during embryonic and early postnatal development.

Table 1

Current Arx mouse models

Model	Mutation	Viability	Spasms	Epilepsy	Brain	Comments	Ref.
Arx^{KO}	Stop codon inserted in exon 2	Perinatal lethal	No data	No data	Gross malformation, DG dysgenesis, CC agenesis, cortical Cb^+ interneurons	Phenotype resembles human XLAG	22
$Arx^{-/Y}$	cKO of exon 2 in interneurons expressing Dlx5/6	≥ 120 days	Only in adult mice; no spasms in infant mice	Myoclonic seizures in males and females at P14-17 and P90-120	No gross malformations; ↓ Cb^+ interneurons	Seizures with features resembling human infantile spasms in adult mice only	43
$Arx^{(GCG)7}$	7 GCG triplets inserted in pA1	≥ 5 mos.	No spasms in infancy noted	Tonic clonic seizures in 1-month-old males, no interictal spikes detected	No gross malformation; ↓ Arx^+ and striatal $ChAT^+$ interneurons	Phenotype resembles human ISSX	44
Arx^{PL}	Pro to Leu substitution at position 355 (353 in humans)*	≥ 5 mos.	No spasms in infancy noted	Increased lethality after bicuculline-induced seizures	No gross malformation; ↓ tangential migration; ↓ striatal $ChAT^+$ interneurons	*Missense mutation in the homeodomain	44
Arx^{PR}	Pro to Arg substitution at position 355 (353 in humans)*	Perinatal lethal	No data	No data	Gross brain malformation; ↓ radial and tangential migration; ↓ striatal $ChAT^+$ interneurons	*Missense mutation in the homeodomain. Phenotype resembles human XLAG	44
$Arx^{(GCG)10+7}$	8 GCT triplets inserted in pA1*	Normal life span	Transient spasms detected between P7 and P11	Frequent spontaneous interictal spikes and electrographic seizures with behavioral arrest	No gross malformation; ↓ cortical Cb^+ interneurons; ↓ striatal $ChAT^+$ and NPY^+ and Cb^+ interneurons	*The expanded mouse pA1 replicates the human-like mutation. Phenotype resembles human ISSX	31

Abbreviations: DG (Dentate Gyrus), CC (Corpus Calosum), Cb (Calbindin), NPY (Neuropeptide Y), ChAT (Choline Acetyl Transferase), ↓ (decrease in expression)