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ARX POLYALANINE EXPANSION MUTATIONS LEAD TO MIGRATION IMPEDIMENT IN THE ROSTRAL CORTEX COUPLED WITH A DEVELOPMENTAL DEFICIT OF CALBINDIN-POSITIVE CORTICAL GABAERGIC INTERNEURONS

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Abstract—The *Aristaless-related homeobox gene* (*ARX*) is indispensable for interneuron development. Patients with *ARX* polyalanine expansion mutations of the first two tracts (namely PA1 and PA2) suffer from intellectual disability of varying severity, with seizures a frequent comorbidity. The impact of PA1 and PA2 mutations on the brain development is unknown, hindering the search for therapeutic interventions. Here, we characterized the disturbances to cortical interneuron development in mice modeling the two most common *ARX* polyalanine expansion mutations in human. We found a consistent ~40–50% reduction of calbindin-positive interneurons, but not Stt+ or Cr+ interneurons, within the cortex of newborn hemizygous mice ($p = 0.024$) for both mutant strains compared to wildtype ($p = 0.011$). We demonstrate that this was a consequence of calbindin precursor cells being arrested or delayed at the ventral subpallium en route of tangential migration. *Ex-vivo* assay validated this migration deficit in PA1 cells ($p = 0.0002$) suggesting that the defect is contributed by intrinsic loss of *Arx* function within migrating cells. Both humans and mice with PA1 mutations present with severe clinical features, including intellectual disability and infantile spasms. Our data further demonstrated the pathogenic mechanism was robustly shared between PA1 and PA2 mutations, as previously reported including Arx protein reduction and overlapping transcriptome profiles within the developing mouse brains. Data from our study demonstrated that cortical calbindin interneuron development and migration is negatively affected by *ARX* polyalanine expansion mutations. Understanding the cellular pathogenesis contributing to disease manifestation is necessary to screen efficacy of potential therapeutic interventions.

Key words: *ARX*, intellectual disability, epilepsy, interneuron migration, calbindin interneurons, polyalanine expansion mutations.

INTRODUCTION

Disruption on cortical interneuron development results in a range of cognitive disorders (Batista-Brito and Fishell, 2009; Rossignol, 2011; Marin, 2012; Kato, 2015; Jacob, 2016). However, the genetic mechanism(s) that confer interneuron subset maturation, migration and specificity, and the molecular manifestation of the diverse clinical presentations conferred by interneuron deficits are unclear. The *Aristaless-related homeobox gene* (*ARX*) (NM_139058.2) encodes a transcription factor critical for interneuron development (Colombo et al., 2007; Colasante et al., 2008, 2009; Friocourt and Parnavelas, 2010, 2011). Complete loss of *ARX* within the developing subpallium is detrimental for tangential migration of GABAergic interneurons in human (Kitamura et al., 2002; Shoubridge et al., 2010, 2016) and mice (Ogata et al., 2000; Kitamura et al., 2002; Colombo et al., 2007; Fulp et al., 2008; Marsh et al., 2009).

Mutations in *ARX* (MIM: 300382), an X-chromosome gene, contributes to intellectual disability in affected male patients with carrier females generally being asymptomatic (Stromme et al., 2002; Shoubridge et al., 2010). Complete loss of ARX function causes catastrophic brain dysmorphology such as X-linked lissencephaly with abnormal genitalia (XLAG) and hydranencephaly due to the loss of projection neurons and interneurons (Dobyns et al., 1999; Bonneau et al., 2002; Kitamura et al., 2002; Kato et al., 2004). The majority of all *ARX* mutations (>60%) expand the first or second polyalanine tract. Affected males with these mutations invariably present with intellectual disability (Shoubridge et al., 2010; Marques et al., 2015). In particular, 85% of patients with expansions to the first polyalanine tract (referred here as PA1 mutation) display seizures, indicating interneuron development deficit (Marques et al., 2015). Key phenotypic features in patients, including infantile spasm-like movements, elec-

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Abbreviations: Cb, calbindin; ChAT, cholinergic; E2, 17β-estradiol; EdU, 5-ethynyl-2′ deoxyuridine; IZ, intermediate zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Pv, parvalbumin; Str, striatum; VT, ventral telencephalon; XLAG, X-linked lissencephaly with abnormal genitalia.

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trodecremetal discharges, and multifocal EEG spikes are well recapitulated in two independent PA1 mouse models (*Arx*$^{(GCG)7}$ mice in Kitamura et al. (2009) and *Arx*$^{(GCG)10+7}$ mice in Price et al. (2009)) (Kitamura et al., 2009; Price et al., 2009; Beguin et al., 2013). Nonetheless, little is known about the pathogenicity of the most common *ARX* mutations (referred here as PA2 mutation), with at least 10–15% patients presenting with infantile spasms in addition to intellectual disability (Marques et al., 2015). Although a mouse model is available (*Arx*$^{432-455dup}$ mice) (Kitamura et al., 2009), phenotypic data for the PA2 strain, including prevalence of seizures is limited.

Using PA1 and PA2 mice developed by Kitamura et al. (2009), we demonstrated a similar loss of Arx protein abundance within the developing subpallium in both models, suggestive of a partial loss of *Arx* function (Lee et al., 2014). In addition, we have identified, between them, a significant overlap in genome wide transcriptome changes (Lee et al., 2014; Mattiske et al., 2016). Studies of the two independent PA1 mouse models found subtype specific loss of GABAergic interneurons in the adult brain (Kitamura et al., 2009; Price et al., 2009; Olivetti et al., 2014). However, deficits in disparate interneuron subtypes between the two PA1 models were noted (Kitamura et al., 2009; Price et al., 2009; Beguin et al., 2013). The importance of elucidating the interneuron deficit is highlighted by the recent treatment study where early prenatal administration of 17β-estradiol (E2) alleviated seizures and restored specific striatal and cortical interneuron populations in the PA1 mice developed by Price et al. (2009) (Olivetti et al., 2014). No data on interneuron deficits or recovery following treatment of the PA2 mice are available. Despite complete loss of *ARX* completely ablating interneuron tangential migration (Colombo et al., 2007; Fulp et al., 2008; Kitamura et al., 2009; Marsh et al., 2016), the cellular consequences of either PA1 or PA2 mutations have not been clearly demonstrated. Expression of PA1 mutation from 12.5dpc and 14.5dpc fails to rescue interneuron tangential migration in *ARX* null background (Nasrallah et al., 2012). However, given *ARX* expression is present within the developing subpallium from 9.5dpc (Miura et al., 1997), the full *in vivo* impact of PA1, as well as that of the PA2, mutation from this earliest expression in comparison to that of the complete loss of *ARX* is still yet to be elucidated.

This study investigates the primary impact of the two most frequent *ARX* polyalanine expansion mutations on cortical interneuron development in PA1 and PA2 mice developed by Kitamura et al. (2009). Our *in vivo* data suggest that both PA1 and PA2 hemizygous mice share a similar profile in the loss of cortical calbindin-positive (Cb+) interneurons specifically as early as the day of birth (P0). Furthermore, our data demonstrate that the specific loss of Cb+ cortical interneurons is unlikely due to a consequence of changes in cellular proliferation or mitotic cycle exit, but a result of migration impediment.

EXPERIMENTAL PROCEDURES

Mice

The relevant institutional Animal Ethics committees approved all animal procedures. PA1 (RRID: IMSR_RBRC03654) and PA2 (RRIC: IMSR_RBRC03653) heterozygote females were imported from RIKEN Bioresource Centre, Japan (Kitamura et al., 2002). Both mouse strains were maintained in the C57BL/6N-Hsd background. Genotyping of animals was performed as described previously (Lee et al., 2014). For harvesting neural stem cell culture, pregnant dams at 14.5dpc were euthanized by cervical dislocation followed by decapitation of embryos. For interneuron birth-dating assay, heterozygote females were time-mated so that detection of vaginal plug on the morning after mating was assigned as 0.5dpc pregnancy. A single intraperitoneal injection of 50–100 mg/kg of 5-Ethynyl-2′ deoxyuridine (EdU) in saline was delivered to pregnant dams at either 11.5dpc, 13.5dpc and 15.5dpc and tissue collected from newborn pups (P0) or pups at the end of gestation (19.5dpc). For frozen tissue sections, whole mount tissue was prepared as described previously (Lee et al., 2014) and sectioned at 10-μm-thick using Leica CM1900 cryostat. Only hemizygous male mice and respective wildtype littermates were used for analysis.

Immunofluorescence and EdU birth-dating assay

Frozen tissue sections were air-dried for 1 h at room temperature prior to immunofluorescence. All procedures were performed in a humidified chamber to prevent drying of tissue sections. For EdU detection, tissue sections were permeabilized in 1 × PBS + 0.5% Triton for 5 min, rinsed once in 1x PBS, then incubated in EdU reaction mix (200 μL per slide) as per manual of Click-iT® EdU Alexa Fluor555® Imagining Kit (Life Technologies). All sections were processed in the absence of light for immunostaining post EdU detection. Sections were then rinsed in 1x PBS, prior to be incubated with blocking solution (10% horse serum and 10% BSA in 1 × PBS + 0.1% Triton) at room temperature. Primary antibodies were incubated at 4 °C overnight, followed by secondary incubation at room temperature for 2 h then mounted with ProLong Gold Antifade Reagent with DAPI (Life Technologies). All sections were washed with 1 × PBS + 0.01% Tween 20 after each antibody staining. Primary antibodies used: rabbit anti-calretinin (1 in 1000, Millipore AB5054, RRID: AB_2068506); rabbit anti-calbindin (1 in 500, Millipore AB1778, RRID: AB_2068336); rabbit anti-GABA (1 in 500, Sigma A2052; RRID: AB_477652); and goat anti-somatostatin (1 in 500, Santa Cruz sc-7819; RRID AB_2302603). Secondary antibody used: donkey anti-rabbit IgG Alexa488 (1 in 400, Life Technologies A21206).

To visualize cells from neurosphere migration assay, cells were subjected to immunofluorescence staining protocol similar to that for tissue sections described above, with the exception that cells were permeabilized with 1 × PBS + 0.2% Triton then blocked with 3% horse serum in 1 × PBS + 0.05% Tween20 at room temperature. Primary antibody use: mouse anti-β-Tublin Isotype III (1 in 1000, Sigma T5076, RRID: AB_532291). Secondary antibody used: donkey anti-mouse Alexa488 (1 in 400, Life Technologies A21202).

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