Three human ARX mutations cause the lissencephaly-like and mental retardation with epilepsy-like pleiotropic phenotypes in mice

Kunio Kitamura¹,³*, Yukiko Itou¹, Masako Yanazawa³, Maki Ohsawa¹, Rika Suzuki-Migishima³, Yuko Umeki¹, Hirohiko Hohjoh², Yuchio Yanagawa⁴, Toshikazu Shinba⁵, Masayuki Itoh¹, Kenji Nakamura³ and Yu-ichi Goto¹

¹Department of Mental Retardation and Birth Defect Research and ²Department of Molecular Genetics, National Institute of Neuroscience, National Center of Neurology and Psychiatry, ³Mitsubishi Kagaku Institute of Life Sciences, ⁴Department of Genetic and Behavioral Neuroscience, Gunma University Graduate School of Medicine, ⁵Stress Disorders Research Team, Tokyo Institute of Psychiatry

*To whom correspondence should be addressed at Kunio Kitamura.
Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. Tel: +81-42-346-1713; Fax: +81-42-346-1743; Email: kitamura@ncnp.gp.jp or kuniokitamura@gmail.com

‡These authors wish to be known that in their opinion, they should be regarded to contribute equally to this work.

¶Present address: Department of Pediatrics, Graduate School of Medicine, University of Tokyo.
ABSTRACT

ARX (the aristaeless-related homeobox gene) is a transcription factor that participates in the development of GABAergic and cholinergic neurons in the forebrain. Many ARX mutations have been identified in X-linked lissencephaly and mental retardation with epilepsy, and thus ARX is considered to be a causal gene for the two syndromes although the neurobiological functions of each mutation remain unclear. We attempted to elucidate the causal relationships between individual ARX mutations and disease phenotypes by generating a series of mutant mice. We generated three types of mice with knocked-in ARX mutations associated with X-linked lissencephaly (P353R) and mental retardation (P353L and 333ins(GCG)7). Mice with the P355R mutation (equivalent to the human 353 position) that died after birth were significantly different in Arx transcript/protein amounts, GABAergic and cholinergic neuronal development, brain morphology, and life span from mice with P355L and 330ins(GCG)7 but considerably similar to Arx-deficient mice with truncated ARX mutation in lissencephaly. Mice with the 330ins(GCG)7 mutation showed severe seizures and impaired learning performance whereas mice with the P355L mutation exhibited mild seizures and only slightly impaired learning performance. Both types of mutant mice exhibited the mutation-specific lesser presence of GABAergic and cholinergic neurons in the striatum, medial septum and ventral forebrain nuclei as compared with wild type mice. Present findings that reveal a causal relationship between ARX mutations and the pleiotropic phenotype in mice suggest that the ARX-related syndrome, including lissencephaly or mental retardation, is caused by only the concerned ARX mutations without the involvement of other genetic factors.
INTRODUCTION

The forebrain comprises a number of functionally and morphologically distinct types of neurons that, in coordination, regulate various aspects of cognition and behavior. ARX, thought to play an important role in forebrain development, is conserved in vertebrates and the ARX protein contains a prl-type homeodomain, four polyalanine tracts, an octapeptide and C-terminal (aristaless) domains (1, 2). Located at Xp22.13, the ARX gene is considered to be a causative factor for X-linked lissencephaly and mental retardation (3, 4, 5), and more than 60 disease-related ARX mutations have been identified (6, 7, 8). These mutations are classified into two groups: one produces more severe lissencephaly, the other less severe mental retardation symptoms. The former group includes deletion, frameshift, nonsense and missense mutations within the homeobox and is associated with malformation syndromes such as lissencephaly, agenesis of the corpus callosum, and ambiguous genitalia. The latter group includes elongation of the first or second polyalanine tract and missense mutations and is associated with syndromes without obvious malformations such as mental retardation, epilepsy and dystonia. The association of mental retardation with epilepsy is incidental since the patients sometimes had frequent and prolonged seizures, secondary neuronal injury, and side effects of anticonvulsant. ARX mutations, however, provide a direct model for genetic association between epilepsy and mental retardation (9). Thus ARX is uniquely involved in a wide spectrum of neurodevelopmental disorders, ranging from mental retardation to lissencephaly.

Arx is expressed in the forebrain as well as in the testis, pancreas and skeletal muscles (3, 10, 11, 12). It is involved in GABAergic neuron development in the cortex and striatum, in addition to cholinergic neuron development in the striatum, medial septum (MS) and ventral forebrain nuclei (3, 13, 14, 15). Arx has both transcriptional activator and repressor domains (16) and functions as a transcriptional repressor (17). A portion of the genetic cascade of Arx in the forebrain has gradually been uncovered, with Dlx2 and Lmo1/Ebf3/Shox2 having recently been identified as upstream and target genes, respectively (18, 19, 20).

Important aspects to be addressed in the ARX-related syndromes, including lissencephaly and mental retardation, are (a) whether the single ARX mutations per se can cause one of the two syndromes and (b) whether there are specific causal relationships between individual ARX mutations and disease phenotypes. In the present report, we
describe the generation of mice with three different knocked-in mutations associated with the ARX-related syndromes and show that the three mutations are causally involved in the syndromes and that the two mutations involved in the ARX-related mental retardation cause seizure and behavioral impairments through the abnormal development of GABAergic and cholinergic neurons.

RESULTS

Generation and general features of three ARX mutant mice

One of the mutations examined in this study was the mutation of a proline (353 in human ARX) in the conserved YPD (tyrosine-proline-aspartic aid) in the prd-type homeodomain of ARX. In humans, P353L develops X-linked myoclonic epilepsy with generalized spasticity and intellectual disability (OMIM300432), while P353R develops X-linked lissencephaly with ambiguous genitalia (XLAG, OMIM300215) (4, 7). We also studied the addition of seven alanines to the first polyalanine tract, which leads to X-linked infantile spasms syndrome/West syndrome (OMIM308350) (4). We knocked-in the three mutations using homologous recombination; that is, to study the first two mutations, we changed the equivalent proline (residue 355) of mouse Arx to either leucine or arginine, while seven GCG-triplets were inserted at residue 330 of the mouse Arx gene (Fig. 1A, B). Using Southern analysis of genomic DNA from embryonic stem cells, we confirmed that each mutation was correctly introduced into the Arx gene (Fig. 1C, D). Hemizygous mice (Arx<sup>mt/Y</sup>) obtained by crossing female heterozygous mice (Arx<sup>mt/X</sup>) with male wild-type mice (Arx<sup>X/Y</sup>) were used in the subsequent experiments (Arx<sup>mt/Y</sup> mice: Arx<sup>P355R/Y</sup> (abbreviation: Arx<sup>PR/Y</sup>), Arx<sup>P355L/Y</sup> (Arx<sup>PL/Y</sup>), Arx<sup>330ins(GCG)7/Y</sup> (Arx<sup>(GCG)7/Y</sup>) mice).

Arx protein from Arx<sup>PR/Y</sup> and Arx<sup>PL/Y</sup> embryos was the same length as that from the wild-type Arx, whereas the protein from Arx<sup>(GCG)7/Y</sup> embryos had a slightly higher molecular weight than that from the wild-type Arx owing to the addition of seven alanines (Fig. 1E). We examined the expression amounts of the mutated Arx transcripts and Arx proteins using quantitative real-time PCR and semi-quantitative Western blotting, respectively. The amount of Arx transcript from Arx<sup>PR/Y</sup> embryos was approximately three times that of the wild-type Arx (this was also confirmed by the end-point PCR, unpublished data), whereas those from Arx<sup>PL/Y</sup> and Arx<sup>(GCG)7/Y</sup> embryos were approximately equal to that of the wild-type Arx transcript (Fig. 1F). The amount of
Arx protein in the Arx<sup>PR/Y</sup> embryo was approximately the same as in the wild-type Arx, while the protein from the Arx<sup>PL/Y</sup> and Arx<sup>(GCG)7/Y</sup> embryos was 40-60% that of the wild-type Arx; lower levels of protein were also found in another GCG mutant mouse, Arx<sup>432-455</sup>del/Y (Fig. 1G, unpublished data). The low levels may be based on susceptibility to proteolysis of the mutated Arx, while detailed analysis of the mechanisms responsible for expression amounts of Arx transcript and protein in the Arx<sup>PR/Y</sup> embryo is required.

Arx<sup>PR/Y</sup> mice died within one day after birth, as did Arx deficient (Arx<sup>-/Y</sup>) mice (3), whereas Arx<sup>PL/Y</sup> mice lived for more than 6 months. Most of the Arx<sup>(GCG)7/Y</sup> mice died within three months, but some of them survived for five to six months. Neonatal Arx<sup>PR/Y</sup> mice had smaller brains and olfactory bulbs, which were also seen in Arx<sup>-/Y</sup> mice (3), whereas the neonatal brains of Arx<sup>PL/Y</sup> and Arx<sup>(GCG)7/Y</sup> mice were nearly as large as those of Arx<sup>X/Y</sup> mice (Fig. 1H).

**Disturbed development of GABAergic and cholinergic neurons in embryonic and neonatal Arx<sup>PR/Y</sup>, Arx<sup>PL/Y</sup>, and Arx<sup>(GCG)7/Y</sup> mice**

Arx deficiency results in the loss of most of the tangential migration, except for that along the subventricular zone (3). The migration of GABAergic progenitor cells was examined using mutant mice cross-bred with heterozygous glutamate decarboxylase (GAD) 67-GFP (neo) mice, (GAD67<sup>GFP/+</sup> mice) (21, 22), which demonstrated that most of the GABAergic interneuron precursors expressed Arx (Fig. 2A-D). The migration modes in Arx<sup>PR/Y</sup> and Arx<sup>PL/Y</sup> embryos were very different from one another (Fig. 2B, C). Arx<sup>+</sup> GABAergic progenitor cells of Arx<sup>PR/Y</sup> embryos failed to initiate migration at E12.5 (Fig. 2B) and began to migrate only along the subventricular zone of the cortex at E14.5 (inset of Fig. 2B). The total number of Arx<sup>+</sup> cells was 28.5±3.4% (P<0.001, n=3) of that found in wild-type cells in the cortical plate at P0 (Fig. 2F). The Arx<sup>+</sup> GABAergic progenitor cells of Arx<sup>PL/Y</sup> and Arx<sup>(GCG)7/Y</sup> embryos began to migrate at E12.5 (Fig. 2C, D), finally reaching 92.8±8.7% and 88.4±11.2% (P<0.02, n=3), respectively, of the number of wild-type cells found in the cortical plate at P0 (Fig. 2G, H). Thus, the PR mutation associated with XLAG resulted in severe defects in cortical tangential migration from the medial ganglionic eminence (MGE), whereas the PL and (GCG)7 mutations associated with ARX-related mental retardation resulted in only slight impairments.

Arx deficiency results in a thickened subventricular zone of striatum and a loss of GABAergic interneurons in the striatum, suggesting the inhibition of both the radial
migration of GABAergic projection neurons from the lateral ganglionic eminence and the tangential migration of GABAergic interneurons from the MGE (3, 15). Arx+ and MAP2-ventricular and subventricular zones of the striatum were thicker in the ArxPR/Y mice at P0 (* in Fig. 2J1, J2). Furthermore, somatostatin (SST)+ cells, a subtype of GABAergic interneurons, accumulated in the subventricular zone of the ventral striatum (around the nucleus accumbens), but no SST+ cells were detected in the mantle zone of the striatum (Fig. 2N). Thus, both radial and tangential migration was significantly suppressed in the ArxPR/Y mice, and a very similar situation was seen with the Arx-/+ mice (3, 15). Radial migration in the ArxPL/Y and Arx(GCG)7/Y mice, on the other hand, was barely suppressed compared with that in the ArxPR/Y mice at P0 (Fig. 2K1, K2, L1, L2), while tangential migration of striatal SST+ cells was suppressed in a manner dependent on each mutation (Fig. 2O, P). Thus, the PR mutation induced severe defects in striatum formation, which depends on both the radial and tangential migration of presumptive GABAergic neurons, while the PL and (GCG)7 mutations caused impairments only in tangential migration of interneurons to the striatum.

Arx induces Gbx1 and Lhx8, making it essential for the development of cholinergic neurons; no cholinergic neurons were detected in the forebrain of the Arx-/- mice (15). In ArxPR/Y and Arx(GCG)7/Y mice, no Lhx8 expression was found in the striatum at P0 (Fig. 2R, T), and only a slight reduction in Lhx8 expression was detected in the striatum of ArxPL/Y mice (Fig. 2S). ARX was expressed in the MS and the vertical limbs of the nucleus of the diagonal bands (DBv) of ArxX/Y mice at P0 (Fig. 2U), while no expression was seen in ArxPR/Y mice (Fig. 2V). Furthermore, the ArxPL/Y and Arx(GCG)7/Y mice showed much reduced ARX expression in the MS and DBv (Fig. 2W, X). These Arx expression patterns in the MS and DBv of the three mutants were also seen for Lhx8 expression (data not shown).

Arx-/- mice with a nonsense mutation that causes XLAG show a thinner cortical plate without a severely abnormal structure (such as inversion of cortical layers) at P0 (3). ArxPR/Y mice with the missense mutation that causes XLAG also exhibited a thinner cortical plate compared with ArxX/Y mice at P0 (85.2±4.8% of wild type, P<0.01, n=3. Fig. 3A, B) The ratio of Tbr1+ deep layer (23) to whole cortical plate in ArxPR/Y mice was slightly higher than that of the wild type (Fig. 3G, H), whereas the Foxp1+ middle layer (24), located at the upper side of the cortical plate, exhibited no clear middle layer structure compared to wild type (Fig. 3E, F). Furthermore, the Satb2+ cells (25) were
packed together in the uppermost layer, resulting in a thinner Satb2+ upper layer (Fig. 3C, D). Thus, the abnormal cortical structure in Arx^{PR/Y} mice at P0 suggests that fine regulation in both the proliferation of neuroepithelial cells in the ventricular zone (3) and the inside-out migration of post-mitotic neurons may be perturbed by Arx^{PR/Y} protein expression in the embryonic ventricular/subventricular zones. Furthermore, this perturbation may be one reason why three cortical layers are formed in the human cortex with XLAG (26, 27). No thin cortical plate or abnormal layer structure was observed in the Arx^{PL/Y} and Arx^{(GCG)7/Y} mice containing mutations that cause mental retardation (data not shown).

The addition of seven alanines to the first polyalanine tract of Arx in vitro results in the formation of intranuclear inclusions and increased apoptosis (28, 29), whereas the present in vivo study using Arx^{(GCG)7/Y} embryos showed that no specific formation of intranuclear inclusions occurs in the migratory Arx+ cells from ganglionic eminence at E12 and in the cortical Arx+ cells at P0 (Supplementary Material, Fig. S1A-D). Furthermore, no increased apoptosis was detected in the ganglionic eminence at E12 (Supplementary Material, Fig. S1E, F). These observations were also seen in another mutation type in the GCG tracts, the duplication of polyalanine tract, “432-455dup” (unpublished data). The absence of intranuclear inclusions in the present in vivo study could be based on differences in the in vivo and in vitro solubility/stability of Arx protein with extra alanine tracts.

In summary, the phenotype of mice with the PR mutation was entirely different from that of mice with the PL and (GCG)7 mutations; this strongly suggests that both groups of mutations can cause specifically developmental abnormalities in ARX-related lissencephaly (XLAG) or mental retardation. In order to further understand mutation effects on ARX-related mental retardation, we focused on the seizure, behavioral and neuronal analysis of the Arx^{PL/Y} and Arx^{(GCG)7/Y} mice at the postnatal stage.

**Epilepsy in the Arx^{PL/Y} and Arx^{(GCG)7/Y} mice**

Epilepsy is a common symptom in ARX-related mental retardation, and we examined the presentation of the epilepsy in the both types of mice. In the behavioral monitoring experiment, seizures were noted in 70% (7/10) of the Arx^{(GCG)7/Y} mice at P1m (one-month-old mice), and one of them (1/10) died during status epilepticus. All seizures started with trembling of the limbs and progressed to tonic clonic convulsions, running
fits and then complete loss of postural control and movement (Supplementary Material, Movie). Some of the mice exhibited hand-washing motions at the end of the seizures. Histologically, ectopically induced NPY expression in mossy fibers of the dentate gyrus was found in the brains of Arx\(^{(GCG)7/Y}\) mice presenting seizures (Fig. 4A1, A2).

In three pairs of Arx\(^{(GCG)7/Y}\) mice and their wild-type littermates, electroencephalographies (EEGs) were recorded in the frontal cortex, hippocampus and striatum together with the behavioral monitoring (Fig. 4B1, B2). In all three Arx\(^{(GCG)7/Y}\) mice, the ictal EEG showed abnormal activities, although no spikes were found during the interictal period (Fig. 4C). At the beginning of the seizure, EEG showed positive spikes in the hippocampus and negative spikes in the frontal cortex and striatum, followed by diffuse spike bursts (Fig. 4D). The first spikes in 26 of the 29 seizures were seen simultaneously in the hippocampus, frontal cortex and striatum. Then 20-30 Hz spike bursts with waxing and waning appeared in the hippocampus, and there were continuous bursts of very high-voltage spikes in the striatum. Finally, the bursts abruptly changed to long-lasting, low-voltage activity with synchronous rhythmic theta waves. Seizures often occurred in clusters, and the spikes were found between the seizure clusters.

In contrast, no Arx\(^{PL/Y}\) mice died during the behavioral monitoring. Only one tonic seizure was seen at P1m in the Arx\(^{PL/Y}\) mice (1/10), and the seizure was of shorter duration (30 seconds) than those seen in the Arx\(^{(GCG)7/Y}\) mice (146±57.9 seconds) but no seizures were observed in Arx\(^{PL/Y}\) mice during EEG recordings. To determine the threshold of seizure and the relationship between age and seizure, the Arx\(^{PL/Y}\) and Arx\(^{X/Y}\) mice were challenged with bicuculline, a GABA\(_A\) receptor inhibitor, at P1m and P3-5m at a relatively low dosage (1.5mg/kg). Myoclonic jerks were observed in most of the mice with both genotypes at both P1m and P3-5m, while generalized seizures developed less frequently in the Arx\(^{X/Y}\) mice at P3-5m (Table 1A). Furthermore, significant differences were noted in the time from injection to onset of jerks and generalized seizures as well as in the number of deaths at both P1m and P3-5m (Table 1A, B). Thus, the seizure threshold was significantly lower in the Arx\(^{PL/Y}\) mice than in the control Arx\(^{X/Y}\) mice.

**Impaired learning performance in both the Arx\(^{PL/Y}\) and Arx\(^{(GCG)7/Y}\) mice**

Learning and memory of both types of mice were tested using a step-through passive avoidance task and two tasks on the eight-arm radial maze. A step-through
passive avoidance task determines whether mice can retain an aversive memory of electric shocks. Naïve Arx^{PL/Y} and Arx^{GCG/7/Y} mice showed no significant difference in latency to enter a darkened chamber compared with Arx^{XY/Y} mice (P=0.098 and P=0.792, respectively, Mann-Whitney’s U-test), which indicated that mobility and motivation for entry were not affected (Fig. 5A, B). After the mice had experienced shocks, latency for entry was significantly shorter in both Arx^{PL/Y} and Arx^{GCG/7/Y} mice than in Arx^{XY/Y} mice (P=0.021 and P<0.001, respectively, Mann-Whitney’s U-test), with a greater reduction of latency seen in Arx^{GCG/7/Y} mice (Fig. 5A, B). These results indicated that neither type of mutant mouse could successfully acquire avoidance behavior through aversive experience.

The win-shift task on the eight-arm radial maze is a well-known hippocampus-dependent spatial learning task. To efficiently obtain all eight pellets at the end of each arm, mice must retain the spatial locations of pellet-retrieved arms within a trial. Performance was evaluated for accuracy, measured as the percentage of chosen arms that were baited. Arx^{PL/Y} mice showed significantly improved accuracy with training, but their accuracy was slightly lower than that of Arx^{XY/Y} mice (genotype effect, F[1,17]=9.12, P<0.01; training effect, F[5,85]=33.1, P<0.0001; genotype x training interaction, F[5,85]=0.702, P=0.624) (Fig. 5C). In Arx^{GCG/7/Y} mice, the accuracy was significantly poorer than in Arx^{XY/Y} mice (genotype effect, F[1,17]=84.3, P<0.0001; genotype x training interaction, F[3,51]=10.8, P<0.0001), as the Arx^{GCG/7/Y} mice showed no significant improvement in accuracy with training (F[3,18]=0.302, P=0.824, one-way RMANOVA) (Fig. 5D). These results indicate that both mutants showed inaccurate performance in pellet retrieval, with the Arx^{GCG/7/Y} mice showing more severe impairment than the Arx^{PL/Y} mice. Because neither mutant used a non-spatial egocentric strategy by visiting each of the eight arms in turn (Supplementary Material, Fig. S2), disabled spatial learning was thought to underlie the inaccurate performance of each type.

The win-stay task of the eight-arm radial maze is designed to evaluate striatum-dependent procedural learning in rodents. Mice can obtain a reward whenever they approach a lit arm. The arrangement of four lit arms is changed for every trial to avoid interfering with spatial memory. After acquiring a manner of approaching lit arms, mice approach the unlit arms less frequently. Arx^{PL/Y} mice showed no significant difference in the ratio of unlit arm choices to lit arm choices compared with the Arx^{XY/Y} mice (genotype effect, F[1,22]=1.78, P=0.20; genotype x training interaction,
F[7,154]=0.43, P=0.88) (Fig. 5E). In contrast, $Arx^{GCG/7/Y}$ mice showed a significantly higher unlit-to-lit ratio throughout training than the $Arx^{X/Y}$ mice did, although they did show a significantly decreased ratio with training (genotype effect, F[1,9]=31.2, P<0.001; training effect, F[4,36]=13.4, P<0.001; genotype x training interaction, F[4,36]=0.19, P=0.94) (Fig. 5F). Thus, we concluded that the $Arx^{GCG/7/Y}$ mice failed to acquire an association between the stimulus of light and the response of approaching, indicating a deficit in procedural learning.

In addition to several kinds of learning disabilities, we confirmed the impaired motor coordination in $Arx^{PL}$ and $Arx^{GCG/7/Y}$ mice and the increased locomotor activity and anxiety-like behavior in $Arx^{GCG/7/Y}$ mice (Supplementary Material, Figs. S3, S4). Increased locomotor activity and anxiety-like behavior are thought to affect learning indices; however, the above-mentioned results in learning tasks were statistically independent from the increased locomotor activity and anxiety-like behavior in $Arx^{GCG/7/Y}$ mice (Supplementary Material, Table S1).

Slight and severe reduction of GABAergic neurons in the cortex and striatum, respectively, of $Arx^{PL/Y}$ and $Arx^{GCG/7/Y}$ mice at P1m

We examined the postnatal distribution of GABAergic and cholinergic neurons based on the occurrence of seizure and impaired learning performance. In addition to being expressed during the embryonic period, Arx is expressed in each subtype of GABAergic interneurons, such as the parvalbumin (PV)$^+$, SST$^+$, and neuropeptide Y (NPY)$^+$ cells and choline acetyltransferase (ChAT)$^+$ cholinergic neurons in the postnatal period (Supplementary Material, Figs. S5). Furthermore, the findings at P1m described in this and the following sections were also confirmed at P2m (data not shown); thus, they were not due merely to a developmental delay.

We examined the number of GAD67$^+$ neurons and subtypes of GABAergic interneurons in the somatosensory cortex of $Arx^{PL/Y}$ and $Arx^{GCG/7/Y}$ mice at P1m. GAD67$^+$, SST$^+$, NPY$^+$ and PV$^+$ interneurons exhibited no severe reduction in the cortex of $Arx^{PL/Y}$ and $Arx^{GCG/7/Y}$ mice, with some exceptions, as suggested by the tangential migration of Arx$^+$ GABAergic neurons during the embryonic and neonatal stages (Supplementary Material, Fig. S6, Fig. 2C, D, G, H). Tangential migration of striatal SST$^+$ cells was suppressed in a manner dependent on each mutation at P0 (Fig. 2O, P). This observation suggested that these subtypes may be reduced at postnatal stages and
there were clearly fewer SST+, NPY+ and NOS (NO synthase)+ interneurons in the striatum of the Arx\(^{PL/Y}\) and Arx\(^{(GCG)7/Y}\) mice (Fig. 6A-I, M). On the other hand, there was only a slight reduction in the number of PV+ interneurons in both mutants as compared with the other subtypes (Fig. 6J-L, M). Furthermore, we found that SST+, NPY+ and NOS+ interneurons were localized to the ventral region and were rarely detected in the dorsal region of the striatum, whereas no regional differences were seen for PV+ interneurons (Fig. 6A-L). Thus, the number of SST+, NPY+ and NOS+ interneurons in the striatum of both mutants was significantly reduced as compared with that seen in the cortex, suggesting that striatal tangential migration was specifically suppressed in both mutants. We also found a subtype-specific reduction in the GABAergic interneurons in the basolateral amygdala of both mutants (Supplementary Material, Fig. S7).

**Reduction of cholinergic neurons in the striatum, medial septum and ventral forebrain nuclei of Arx\(^{PL/Y}\) and Arx\(^{(GCG)7/Y}\) mice at P1m**

Striatal ChAT+ cholinergic interneurons were reduced by 50.1±6.8% (P<0.001, n=3) in the Arx\(^{PL/Y}\) mice, whereas no ChAT+ interneurons were found in the Arx\(^{(GCG)7/Y}\) mice, as expected from the Lhx8 expression at P0 (Figs. 7B, C, 2S, T). Arx\(^{432-455dup/Y}\) mice also had no ChAT+ interneurons in the striatum (unpublished data). This observation suggests that migration and differentiation of cholinergic interneurons are fully suppressed by the elongation of the first and second polyalanine tracts and only partially by the PL mutation.

Forebrain cholinergic projection neurons are found in the MS (Ch1), DBv, and DBh (Ch2 and Ch3) and the basal magnocellular complex (Ch4), which is composed of the magnocellular preoptic nucleus (MCPO), substantia innominata, ventral pallidum and basal nucleus (NB). Ch1 and Ch2 provide major innervation to the hippocampus; Ch3 innervates the olfactory bulb; and Ch4 innervates the cortex. ChAT+ projection neurons were significantly reduced, to 53.2±4.8% of the wild-type level in the MS of the Arx\(^{PL/Y}\) mice (P<0.001, n=3, Fig. 7E), and were even more severely reduced in the Arx\(^{(GCG)7/Y}\) mice (23.8±4.0%, P<0.001, n=3, Fig. 7F). Furthermore, the ChAT+ neurons of Arx\(^{PL/Y}\) and Arx\(^{(GCG)7/Y}\) mice were reduced more severely in the DBv than in the MS (Fig. 7E, F). GAD67+ neurons, another component of both the MS and DBv, were reduced in the Arx\(^{PL/Y}\) mice and were not found in the Arx\(^{(GCG)7/Y}\) mice, except for some expression in the most anterior MS and DBv (Fig. 7H-I and inset of I). The number of ChAT+ neurons
in the MCPO of both mutants was decreased to approximately 70% of the wild-type level (80.1±8.5%, P<0.01, n=3 for Arx<sup>PL/Y</sup> mice, 68.4±7.5%, P<0.001, n=3 for Arx<sup>(GCG)7/Y</sup> mice, Fig. 7J-L), and the number of neurons in the NB of both mutants was also reduced (data not shown). Furthermore, we detected acetylcholinesterase (AchE)<sup>+</sup> axonal arbors of cholinergic projection neurons in the hippocampus and cortex, which are innervated by cholinergic projection neurons in the medial septum and basal magnocellular complex, respectively. A clear reduction in the AchE<sup>+</sup> axonal arbors was noted in the hippocampus and somatosensory cortex of Arx<sup>PL/Y</sup> mice (Fig. 7N, Q, S) compared to Arx<sup>X/Y</sup> mice, in addition to a severe reduction seen in Arx<sup>(GCG)7/Y</sup> mice (Fig. 7O, R, S). These data indicate aberrant formation of the Ch1/Ch2 (septohippocampal) and C4 systems in both mutant strains.

**DISCUSSION**

**Diversity of ARX mutations and ARX-related lissencephaly and mental retardation with epilepsy**

One question to be addressed regarding the diversity of ARX-mutations in the ARX-related lissencephaly (XLAG) and mental retardation is whether the conditions are caused by individual mutations without involvement of any other genetic factors. We addressed this topic by introducing PR, PL and (GCG)7 mutations into the mouse genome. We found that phenotypes of Arx<sup>PR/Y</sup> mice are very similar to those of Arx<sup>-/Y</sup> mice with a truncated ARX mutation in XLAG (3, 15). The abnormal cortical layer formation, abnormal structure of the striatum and deficiency of GABAergic neurons in the cortex and striatum caused by PR mutation closely mimic XLAG (5, 26, 27). Still, the mechanisms underlying the abnormal cortical formation specific to XLAG are not yet clear due to the perinatal death of both mouse models of XLAG (30, 31). On the other hand, two structurally different mutations associated with ARX-related mental retardation, including the single amino acid replacement (PL) and elongation of polyalanine tract ((GCG)7), also caused a common phenotype of ARX-related mental retardation with epilepsy. Thus, the present study strongly suggests that PR and PL/(GCG)7 mutations independently cause lissencephaly and mental retardation, respectively, without the involvement of any other genetic factors.

The importance of the conserved YPD in the homeodomain was demonstrated in the Arx<sup>PR/Y</sup> and Arx<sup>PL/Y</sup> mutant mice (4, 7). Proline in the YPD is predicted to have
influence on homeodomain structure by providing the proper hydrophobic environment (32), while leucine and arginine have stronger and much weaker (hydrophilicity) hydrophobicity compared with proline, respectively (33). Thus, the hydrophobic environment suitable for the normal homeodomain structure and function may be destroyed by each mutation. It was surprising that the (GCG)7 mutation did not result in the specific formation of intranuclear inclusions and apoptosis that were observed in the \textit{in vitro} functional test of the mutation (28, 29) and this was also true of the $Arx^{432-455dup/Y}$ mice in our present study (unpublished data). The elongation of the polyalanine tract may function in the downregulation of ARX activity in cooperation with other functional domains of ARX such as homeodomain.

\textbf{Seizure and ARX-positive neurons}

Seizures occurred in most of the $Arx^{(GCG)7/Y}$ mice at P1m, and approximately 10\% died during an episode of status epilepticus, suggesting that the seizure caused the death. In contrast, only one $Arx^{PL/Y}$ mouse exhibited seizures, which were mild and occurred at one month of age. The observation that the longevity of $Arx^{PL/Y}$ mice was not different from that of $Arx^{XY}$ mice clearly shows that the seizure phenotype of the $Arx^{PL/Y}$ mice was milder than that of the $Arx^{(GCG)7/Y}$ mice. The onset latency and characteristics of the seizures in the $Arx^{PL/Y}$ mice following a low dose of bicuculline were significantly different from those of the $Arx^{XY}$ mice, suggesting that the $Arx^{PL/Y}$ mice had the epileptic phenotype. We conclude that both mutants show the epileptic phenotype that becomes apparent during late infancy to weaning age, albeit with different levels of severity in the two mutants. Interestingly, both types of mutants showed the epileptic phenotype with a severity of seizures that was similar to that in the corresponding human types. Most patients with the (GCG)7 mutations are diagnosed with infantile syndrome, whereas patients with the PL mutation develop myoclonic epilepsy (4). Epilepsy in patients with the (GCG)7 mutation is more severe than that in patients with the PL mutation.

Recently, it has been shown that specific abnormalities in the development or function of cortical GABAergic interneurons are linked to epilepsy (34, 35). $Arx^{-/Y}$ mice exhibit interneuron migration deficiencies (3), and the $Arx^{PR/Y}$ mice in our present study were identical or very similar to $Arx^{-/Y}$ mice in this regard. Although $Arx^{-/Y}$ and $Arx^{PR/Y}$ mice did not exhibit seizures because they died around birth, the insufficient GABAergic interneuron migration may conceivably cause epilepsy in patients with lissencephaly due
to the PR mutation. Surprisingly, we found that cortical and hippocampal GABAergic interneurons did not show a marked reduction in the $Arx^{PL/Y}$ or $Arx^{(GCG)^7/Y}$ mice, but these mice did have seizures. Previous studies have shown that septal GABAergic projection neurons innervate GABAergic interneurons, and their cholinergic projection neurons form synapses with all neuron types in the hippocampus (36, 37). It should also be noted that abolishing septal cholinergic projection neurons facilitated kindling in the hippocampus (38) and that inhibiting GABA A receptors expressed in GABAergic and cholinergic projection neurons in the septal region induced epileptiform activity in the hippocampus and seizures (39). Our results with the septum raise the possibility that seizures in $Arx^{PL/Y}$ and $Arx^{(GCG)^7/Y}$ mice are caused by a loss of cholinergic and GABAergic projection neurons in that region.

We also revealed the loss of the GABAergic interneurons in the striatum. Much experimental evidence has suggested that the basal ganglia play an effective role in controlling seizures in animal epilepsy models (40, 41), but the effects of the loss of striatal GABAergic interneurons in epilepsy remain enigmatic. We recorded EEGs in the striatum to examine its involvement in seizure generation and found that spike and burst patterns in the striatum were maintained at high voltage even after the voltage decayed in the hippocampus. Bipolar recording in the striatum also showed high-amplitude waves during the seizures, indicating local generation of epileptic activity. These EEG findings support a significant role of the striatum in seizure generation, although we could not detect the primary locus. The striatum receives input from large cortical areas. Epileptiform activity in the cortex evokes depolarizing bursts that are accompanied by action potentials in striatal projection neurons (42), and neocortical seizure-like EEG oscillations are evoked by tetanization of the striatum (43). Our results suggest that the loss of the GABAergic interneurons that inhibit projection neurons in the striatum may play a role in the epileptic network, inducing status epilepticus.

**Impaired learning performance and ARX-positive neurons**

Both $Arx^{PL/Y}$ and $Arx^{(GCG)^7/Y}$ mice showed defects in several aspects of learning and memory. Passive avoidance is used as an experimental model of amnesia in rodents treated with cholinergic antagonists in the forebrain or striatum (44). Neither mutant could successfully acquire avoidance behavior, and the $Arx^{(GCG)^7/Y}$ mice, with their severely impaired cholinergic projection neurons and interneurons, showed a more...
prominent defect than the $Arx^{PL/Y}$ mice. This observation suggests that these memory defects are due to cholinergic dysfunction in the forebrain of (GCG)7 and PL mutant mice. The win-shift task of the radial maze is a hippocampus-dependent spatial working or episodic-type memory task (45). Both types of mutant mice showed inaccurate performance on this task, with a more severe effect seen in the $Arx^{GCG/7/Y}$ mice, which did not improve their performance with training. Significant impairment in win-shift performance was produced not by a single lesion in the cholinergic projection neurons in Ch1/2 but by a complex lesion in either the GABAergic projection neurons in Ch1/2 or the cholinergic projection neurons in Ch4 (46). Both types of mutant mice had a coincident reduction or loss of both projection neurons in Ch1/2 and cholinergic projection neurons in Ch4, which likely underlies their inaccurate performance. In the $Arx^{GCG/7/Y}$ mice, this reduction or loss was more severe than in the $Arx^{PL/Y}$ mice. Moreover, a reduction in the hippocampal PV+ interneurons could have affected win-shift performance (47) in the $Arx^{GCG/7/Y}$ mice (data not shown, Supplementary Material, Fig. S6). Thus, these histological differences from the $Arx^{PL/Y}$ mice may have been manifested in the lack of improvement in win-shift performance of the $Arx^{GCG/7/Y}$ mice. Win-stay performance, which represents striatum-dependent procedural learning (45), was impaired only in the $Arx^{GCG/7/Y}$ mice in the present study. Cholinergic interneurons, which were reduced in both mutants, play an important role in procedural learning (48, 49). The complete loss of the striatal cholinergic interneurons in $Arx^{GCG/7/Y}$ mice likely led to their inferior win-stay performance. On the other hand, $Arx^{PL/Y}$ mice showed a less severe reduction in striatal cholinergic interneurons and unimpaired win-stay performance.

In conclusion, we reported a causal relationship between three $Arx$ mutations and the pleiotropic phenotype using three generated mutant mice. The present mutant mice offer various research opportunities, including elucidation of the molecular properties of Arx cascade affected by each mutation and pathogenesis of aberrant neural systems caused by reduction of GABAergic and cholinergic neurons in the forebrain. Thus, these mutant mice could serve as excellent models for research on remarkable pleiotropy of ARX-related lissencephaly and mental retardation with epilepsy and, moreover, for reversing the syndromes (50).
MATERIALS AND METHODS
The experiments were conducted in accordance with the animal care regulations of the Mitsubishi Kagaku Institute of Life Sciences, the National Institute of Neuroscience and the Tokyo Institute of Psychiatry.

Construction of the targeting vectors. We constructed three types of targeting vectors in which partial Arx fragments (3) containing the different mutations were linearly ligated to the Neo and DTA genes; these DNA fragments were then subcloned into pBluescript II (Figs. 1A, B). The P355R mutation (CCT --> CgT) was introduced using the GENEEditor TM in vitro Site Directed Mutagenesis System (Promega, WI) and the sense primer 5’-CCAGCGGGAGGAACCTcGACGGGCTTTCAAGACGCACTACC-gTGACGTCTTCAACCCAGG-3’ (the italicized bold lowercase letter shows the mutation site, and the newly introduced XhoI site is underlined) (Fig. 1A). The P355L (CCT --> CcT) mutation was introduced by PCR using the sense primer 5’-GGCTGCAGCGGGCGGCGGCG-3’ and the antisense primer 5’-AAGACGTCA-aGGTAGTGCATTTCTTGGAAGCAGCCCGCTcGAGTTCCCTCCAGCTGGTAA-3’ (the italicized bold lowercase letter shows the mutation site, and the newly introduced XhoI site is underlined) (Fig. 1A). The addition of seven alanines into the first polyalanine domain was carried out by inserting 7x(GCG) into a NotI site (325GCGGCCGC332), designated as the “330ins(GCG)7” mutation (GCCGC --> GCgggccggccggccggccggccc-gGC); this mutation was introduced by PCR using the sense primer 5’-GGCTGCAGCGGGCGGCGGCGGC-3’ and the antisense primer 5’-AAGACGTCAAGAGCACTGGG-3’ (the italicized bold lowercase letters show the insertion sequence, and the destroyed NotI site is underlined) (Fig. 1B).

Transfection, screening and generation of three Arx mutant mice. To obtain the targeted embryonic stem (ES) cells, we used a positive-negative selection strategy in the CCE ES cells (a gift from Dr E. Robertson), which are derived from 129/Sv. We screened ES colonies by Southern blot analysis with a 5’ probe and a 3’ probe external to the genomic sequences contained in the targeting vector and a Neo gene (red and blue bars in Fig. 1A, B). Furthermore, we confirmed the introduction of each mutation by Southern blot analysis using the introduced XhoI site in the PL and PR mutations or the lost NotI
site in the (GCG)7 mutation (green bar in Fig. 1A, B). We injected the positive ES clones into (C57BL/6J × BDF1) F1 or C57BL/6J blastocysts, which we then transferred into pseudopregnant female recipients. The resulting chimeric mice were bred with C57BL/6J females. Transmission of the targeted Arx locus was confirmed by Southern blot patterns. We obtained three Arx mutant mice (Arx<sup>mt</sup>/Y: Arx<sup>PR</sup>/Y, Arx<sup>PL</sup>/Y, Arx<sup>(GCG)7</sup>/Y), which were used in the subsequent analysis by crossbreeding with Arx<sup>mt</sup>X and Arx<sup>X</sup>/Y mice. The Arx<sup>mt</sup>/Y hemizygotes derived from two independent targeted ES cell lines were phenotypically indistinguishable.

**Genome DNA, Arx transcript and protein analysis.** For Southern blots, we analyzed aliquots of genomic DNA from ES cells or tails of mice by 35P-radioactive filter hybridization (3). For quantitative real-time PCR analysis, we carried out oligo-dT-primed reverse-transcription on aliquots of total RNA from the forebrain at E14.5 and used the single-strand cDNA products. Quantitative real-time PCR was performed with TaqMan primers on an ABI 7300 Real Time PCR system (Applied Biosystems, CA). All samples were analyzed in triplicate and were normalized relative to glyceraldehyde-3-phosphate dehydrogenase (Gapdh) expression, which served as an internal control for each sample. Gapdh expression was monitored using TaqMan rodent Gapdh control reagents (Applied Biosystems). We analyzed Arx expression using TaqMan Arx primers (Applied Biosystems). Expression levels were normalized to the wild type for comparison. Data was analyzed with SDS 2.0 (Applied Biosystems) and Microsoft Excel, using the Ct method and the expression level of Gapdh as an internal reference for each sample. We carried out western blot analyses of RIPA buffer-extracted samples using an ARX polyclonal antibody (3). All samples were analyzed in triplicate and were normalized relative to actin expression, and the expression levels were normalized to the wild type for comparison. Expression was detected by chemiluminescence (ECL, Amersham/GE Healthcare, NJ), and the data were analyzed using the publicdomain Image J program developed by the U.S. National Institutes of Health.

**Histological analysis.** Embryos were immersed and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline for 12 hours, and postnatal animals were fixed by cardiac PFA perfusion. Brains were post-fixed overnight, cryoprotected in a gradient of
sucrose to 30%, frozen in embedding medium (OCT, Tissue-Tek) and serially sectioned at a thickness of 10 μm using a cryostat. For immunohistochemical staining, in situ hybridization and acetylcholinesterase histochemistry, four to six semi-sequential coronal sections per region (striatum, MS, MCPO, sensory cortex, hippocampus and basolateral amygdala) of three or four wild type or mutant mice were selected according to the mouse brain atlas (51). Immunohistochemical staining and in situ hybridization were carried out as described (3). The antibodies used were anti-ARX antibody (3), anti-PV antibody (Sigma), anti-ChAT antibody (Chemicon/Millipore, MA), anti-NOS antibody (Chemicon), anti-MAP2 antibody (Chemicon), anti-TBR1 antibody (abcam), anti-Foxp1 antibody (abcam) and anti-SATB2 antibody (Bio Matrix Research Inc). Immunohistochemical signals were visualized by the following systems: (a) peroxidase-labeled secondary antibody/diaminobenzidine, (b) GFP-GAD67 and an anti-ARX antibody intensified with Alexa594-streptavidin (Molecular Probes/Invitrogen, CA), and (c) an antibody intensified with FITC-labeled secondary antibody (Chemicon) and anti-ARX antibody intensified with Alexa594-streptavidin (Molecular Probe). The antisense probes were Lhx8 (a gift from Dr V. Pachnis), SST, NPY, and GAD67. Acetylcholinesterase histochemistry was performed as described previously (52). In each section, the number of positive cells per square micrometer in the observed region was calculated. Each value from the mutant mice was normalized to the wild-type mouse. Statistical analysis was done using a Student’s t test. Graphs show mean ± S.E.M.

**Video monitoring of mice.** Video monitoring was conducted for Arx\(^{PL/Y}\) mice at the onset of weaning (P1m) and at the adult age (P5-6m), and for Arx\(^{(GCG)}7/Y\) mice at P1m (each n=10) in their home cages. The room light was on from 8:00 A.M. to 8:00 A.M., and their behavior was monitored with a CCD camera during the dark period (9-11 hr/day) for 4 days under infrared light illumination. The data were stored in a hard disk recorder, and convulsive behavior was checked off-line by viewing the animals’ behavior on a screen.

**Surface and depth EEG recording.** Arx\(^{PL/Y}\) and Arx\(^{(GCG)}7/Y\) mice and their wild-type littermates (n=3 for each group) at P39-49 (P1m) were used for EEG recordings. Under chloral hydrate anesthesia (400 mg/kg, ip), four stainless wire electrodes (200 μm in diameter) were implanted using a stereotaxic apparatus (SR-8N, Narishige, Japan). One electrode was placed on the dura at the frontal cortex (F-cx; AP +1.0, L 2.0 from the
Bregma; 51). Two wire electrodes were placed in the dorsal (Str-u; AP +1.0, L 2.0, DV 3.0) and ventral (Str-l; DV 4.0) parts of the striatum. Another wire electrode was inserted into the hippocampus (Hip; AP –2.0, L 2.0 DV 2.0) (Fig. 3B1, B2). The reference screw electrode was placed on the cerebellum surface. Lead wires from the electrodes were connected to a socket. And the electrodes and the socket were firmly attached to the skull with acrylic resin cement. After a recovery period of 3 days after surgery, an EEG was recorded for 1-2 days in a sound-attenuated experimental box. The data were amplified 1000 times (time constant; 0.1 sec, low-pass filter; 100 Hz, AB-621G, Nihon Kohden, Japan) and monitored on a computer screen (Spike2 ver. 5, Cambridge Electronics Design, UK). The behavior of the mice was monitored with a CCD camera in the experimental box and recorded using a digital video camera (DCR-DVD301, Sony, Japan). The behavioral changes and accompanying EEG data were checked offline. After the experiments, electrode positions were verified histologically (Fig. 3B).

**Bicuculline treatment.** To determine the seizure threshold and its change with age, $Arx^{PL/Y}$ and $Arx^{X/Y}$ mice at P1m (each n=6) and P3-5m (each n=8) received subcutaneous injections of 1.5 mg/kg bicuculline and their convulsive behavior was monitored for 30 min. The number of mice displaying myoclonic jerks and generalized seizures was recorded. In addition, the onset times of the first jerk and the first generalized seizure, and the time of death from injection were recorded. Statistical differences in the number of mice and the time between injection and epileptic events were analyzed by Fisher’s exact test and Mann-Whitney’s U test, respectively.

**Experimental design for behavioral testing.** The following behavioral tests were performed using $Arx^{PL/Y}$, $Arx^{(GCG)^7/Y}$ and $Arx^{X/Y}$ mice backcrossed to a C57BL/6J genetic background for F8 to F9 generations: the wire-hanging test (Supplementary Material, Fig. S3), the rotarod test (Supplementary Material, Fig. S3), the open-field test (Supplementary Material, Fig. S3), the step-through passive avoidance test (Fig. 4), the light-dark transition test (Supplementary Material, Fig. S4) and two divided tasks on the eight-arm radial maze including the win-shift task and the win-stay task (Fig. 4). After weaning around postnatal week 4 (postnatal day 26 to 29), the mice were housed two to five animals to a cage in a room with a 12 hr light/dark cycle (light beginning at 8:00 A.M.) with ad libitum access to food and water, except during the eight-arm radial maze
tests. Behavioral testing began after three nights and was performed between 9:00 A.M and 7:00 P.M. Almost all behavioral tests were carried out using identical mice. The Arx\textsuperscript{(GCG)7/Y} mice had a short life span and were almost all deceased by 10 weeks of age. To avoid a decrease in the number of mice tested due to death, the two tasks on the radial maze were separated from the other tests and were begun as soon as possible after weaning.

**Step-through passive avoidance.** The step-through passive avoidance test was performed over two days using the testing apparatus (O’Hara & Co. Tokyo) as described by Karl et al. (53). On day 1, the mouse was placed into the illuminated chamber facing the door to the darkened chamber. The door then opened, and the latency of entry to the darkened chamber was measured. Electric shocks (0.2 mA) were delivered 3 sec after the mouse entered. The mouse could freely escape from the darkened chamber with the door open and was allowed to explore these chambers for 60 sec after the shocks. On day 2, the mouse was again placed into the illuminated chamber, and the latency of its entry to the darkened chamber was measured, with a 300-sec cut-off time.

**The win-shift task on the eight-arm radial maze.** The win-shift task was performed in a manner similar to that described by Miyakawa et al. (54), using the eight-arm radial maze (O’Hara & Co.). The win-shift task was conducted during a habituation and training phase. Food deprivation (each mouse received only a 2.5- to 3.0-g pellet a day) began three to four days before the habituation phase. Over the seven days of the habituation phase, the mouse underwent each of the three steps of pre-training in turn. In the first step, the mouse was allowed to freely explore the maze for 10 min without food reward. In the second and third steps, the protocol described by Miyakawa et al. (54) was followed. After the habituation phase, actual training began. All eight arms were baited with food pellets. The mouse was allowed to retrieve all eight pellets, with a 7-sec confinement in the central platform before being presented with the next arm choice. The trial continued until the mouse had consumed all eight pellets or 10 min had elapsed. Training occurred once daily for 18 days. Image J RM software was used both to carry out the procedure and to analyze behavioral performance. Data for each mouse were averaged every three trials for statistical analysis.
The win-stay task on the eight-arm radial maze. The eight-arm radial maze was modified for the win-stay task. The eight arms had black opaque walls, and food dispensers were attached to the end of each arm to drop a sucrose pellet into the well. Lightbulbs were set above the entrance to each arm facing the end of the arm. The apparatus was surrounded by a black curtain to restrict spatial cues and room light. The mice underwent the same food-deprivation schedule as in the win-shift task. The habituation and training phases were conducted for the win-stay task, during which four randomly selected arms were lit, but no more than three successive adjacent arms were lit on each trial. In the habituation phase, the mouse was allowed to freely explore the maze for 10 min a day for 3 days. The actual training was performed as described previously (55), following the habituation phase. A single pellet was placed in each well of the lit arms. The mouse was given two chances to retrieve the pellet in each lit arm and confined in the central platform for 7 sec between each lit or unlit arm choice. This procedure was manually operated by remote control with video monitoring. One trial per day continued until the mouse had consumed all eight pellets or 10 min had elapsed. The latency to consume all pellets and the number of lit or unlit arms visited were measured for each trial. Data for each animal were averaged every three trials for statistical analysis.

Collection of behavioral indices for the eight-arm radial maze for the Arx\(^{(GCG)7/Y}\) mice. Behavioral indices were collected, except for trials in which total choices were fewer than eight, which came from the total choices when a mouse retrieved all eight pellets without incorrect arm choices, because irregular and abnormal behavior occurred so often that the mouse had difficulty turning and choosing an arm. Additionally, if we saw an epileptic seizure, we allowed a 30- to 60-minute interval before a trial to let the mouse calm down. Trials were sometimes stopped when it was apparent that a mouse could not move without difficulty.

Image analysis and Statistical analysis. Software used for the behavioral tests (Image J OF, Image J LD, Image J EP and Image J RM) was modified on the public-domain Image J program, developed by the U.S. National Institutes of Health (available through O’Hara & Co.). Each behavioral index was compared between the Arx mutant mice and Arx\(^{XY}\) mice nursed by the heterozygous dams with the same kind of mutant allele. The data were analyzed using a two-tailed Student’s t-test or two-way RMANOVA, unless noted
otherwise, using Excel or GraphPad Prism 5. Graphs show mean ± S.E.M.

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CONFLICTS OF INTEREST STATEMENT

None declared.

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LEGENDS TO FIGURES

Figure 1

Generation and general features of Arx^{PR/Y}, Arx^{PL/Y} and Arx^{(GCG)7/Y} mice

A, B, Schematic representation of the wild-type Arx allele (top), targeting vector (middle) for introducing either the PL or PR mutations (A) and the (GCG)7 mutation (B), and the mutated allele after homologous recombination (bottom). The mutation points are indicated by (*). A new XhoI site is formed upon the introduction of the PL or PR mutations (A), while a NotI site is destroyed upon the introduction of the (GCG)7 mutation (B).

C, D, Southern blot analysis of the targeted ES clone with either the PL or PR mutations (C) and the (GCG)7 mutation (D). Genomic DNA from the ES cells was digested with either Eco RI for the PL/PR mutations (red bar in A) or BglII for the (GCG)7 mutation (red bar in B), and both digests were probed with a 5’-probe (C, D). Genomic DNA was then digested with KpnI for the PL/PR mutations (blue bar in A) or BamHI for the (GCG)7 mutation (blue bar in B), and both digests were probed with a 3’-probe (C, D). To confirm that each mutation was introduced, genomic DNA was either digested with XhoI (green line in A) and probed with a Neo-probe for the PL/PR mutations (C) or digested with BglII/NotI (green bar in B) and probed with a 5’-probe for the (GCG)7 mutation (D).

E, Western blotting of Arx protein from the forebrains of three mutant embryos at E14. Arx protein from Arx^{PR/Y} and Arx^{PL/Y} embryos was the same length as the wild-type Arx whereas the protein from Arx^{(GCG)7/Y} embryos had a slightly higher molecular weight than that from the wild-type Arx.

F, Quantitative analysis of Arx transcript by real-time PCR. All values of the Arx transcript were normalized for Gapdh. The Arx transcript from Arx^{PR/Y} embryos was approximately three times that of the wild-type Arx, while the Arx transcripts of Arx^{PL/Y} and Arx^{(GCG)7/Y} embryos were approximately the same as those of the wild-type Arx.

G, Semi-quantitative analysis of Arx protein by Western blotting. All values of the Arx protein were normalized for actin. Arx protein levels from Arx^{PR/Y} embryos were approximately the same as those from the wild-type Arx, while Arx protein levels in Arx^{PL/Y} and Arx^{(GCG)7/Y} embryos were 66.4±22.6% (P<0.02, n=5) and 48.4±16.4% (P<0.001, n=5) of those in the wild-type Arx, respectively.

H, Dorsal views of whole brains of newborn mice. Arx^{PR/Y} mice had smaller brains and olfactory bulbs, whereas the neonatal brains of Arx^{PL/Y} and Arx^{(GCG)7/Y} mice were almost as large as those of Arx^{X/Y} mice.
Figure 2
Characterization of the cortex (Cx), striatum (Str) and medial septum (MS) of embryonic and neonatal $Arx^{PR/Y}$, $Arx^{PL/Y}$ and $Arx^{(GCG)7/Y}$ mice

A-D, Cortical tangential migration in embryos (E12.5) crossbred with GAD67 $^{GFP/+}$ mice. $Arx^+$ (red)/GAD67-GFP$^+$ (green) cells in the $Arx^{X/Y}$ embryos began to migrate from the ganglionic eminence (GE) (A), while no migration from the GE was seen in the $Arx^{PR/Y}$ embryos (B). Only late migration from the GE along the subventricular zone of the Cx was seen at E14.5 (inset of B). $Arx^+/GAD67$-GFP$^+$ cells in $Arx^{PL/Y}$ and $Arx^{(GCG)7/Y}$ embryos migrated normally, although the number of cells was slightly reduced compared with that in the $Arx^{X/Y}$ embryos (C, D).

E-H, $Arx^+$ cells in the Cx at P0 (E-H). Significantly reduced numbers of $Arx^+$ cells were seen in the $Arx^{PR/Y}$ mice, and some of them were clustered at the subplate (F). $Arx^+$ cells were only slightly reduced in the $Arx^{PL/Y}$ and $Arx^{(GCG)7/Y}$ mice (G, H).

I1-L1 (anterior Str) and I2-L2 (posterior Str), ARX (red) and MAP2 (green) expression in the Str at P0. The $Arx^+$ and MAP2-ventricular zone of the anterior Str of $Arx^{PR/Y}$ mice showed increased thickness (* in J1), while no increase was seen in $Arx^{PL/Y}$ (K1) or $Arx^{(GCG)7/Y}$ mice (L1). The thickening of the ARX$^+$ and MAP2$^+$ ventricular zone was also seen in the posterior Str of $Arx^{PR/Y}$ mice (* in J2), while no increase was seen in the $Arx^{PL/Y}$ (K2) and $Arx^{(GCG)7/Y}$ mice (L2).

M-P, SST$^+$ interneurons in the Str at P0. SST$^+$ interneurons were distributed throughout the Str of the $Arx^{X/Y}$ mice (M), whereas they gathered in the thickened ventricular zone of the striatum of $Arx^{PR/Y}$ mice (* in N) and were not present in the mantle zone of the Str (N). On the other hand, a small number of SST$^+$ interneurons were seen in the mantle zone of the Str of $Arx^{PL/Y}$ mice compared with $Arx^{X/Y}$ mice (O), while most of the SST$^+$ interneurons were seen in the ventricular zone of the Str in $Arx^{(GCG)7/Y}$ mice (P).

Q-T, $Lhx8$ expression in the Str at P0. $Lhx8$ expression was seen throughout the Str of $Arx^{X/Y}$ mice (Q), while no expression was seen in $Arx^{PR/Y}$ mice (R). $Lhx8$ expression was seen in the ventral half of the Str of $Arx^{PL/Y}$ mice (S), whereas no expression was seen in the $Arx^{(GCG)7/Y}$ mice (T).

U-X, $Arx$ expression in the MS and vertical limbs of the nucleus of the diagonal band (DBv) at P0. ARX was expressed in the MS and DBv of the $Arx^{X/Y}$ mice (U), but no expression was seen in $Arx^{PR/Y}$ mice (V). Furthermore, significantly reduced Arx expression was observed in the $Arx^{PL/Y}$ and $Arx^{(GCG)7/Y}$ mice (W, X). MGE: medial ganglionic eminence, GP: globus pallidus. Scale bars: A-D, 100 μm, E-H, 250 μm, I1-T, 500 μm, U-X, 500 μm.
Figure 3

Aberrant cortical layer formation in ArxPR/Y mice

A, B, Cresyl violet staining of the cortical plate of ArxXY (A) and ArxPR/Y (B) mice at P0. The thickness of the cortical plate was 85.2±4.8% of wild type (P<0.01, n=3). C, D, Satb2 imaging of the upper cortical layer of ArxXY (C) and ArxPR/Y (D) mice. Satb2+ cells in ArxPR/Y mice were packed together in the uppermost layer, resulting in a thinner Satb2+ upper layer than that of the wild type. E, F, Foxp1 imaging of the middle cortical layer of ArxXY (E) and ArxPR/Y (F) mice. The Foxp1+ middle layer of ArxPR/Y mice was located at the upper side of the cortical plate and exhibited no clear middle layer structure compared to wild type. G, H, Tbr1 imaging of the deep cortical layer of ArxXY (G) and ArxPR/Y (H) mice. The ratio of the Tbr1+ deep layer to the entire cortical plate in ArxPR/Y mice (60.3±5.3%, p<0.01, n=3) was slightly higher than that of ArxXY mice (48.3±3.8%, p<0.01, n=3). CP, cortical plate; UL, upper layer; ML, middle layer; DL, down layer. Scale bars: A-H, 100 μm.

Figure 4

Presentation of seizures in Arx(GCG)7/Y mice

A1, A2, Ectopic NPY expression in mossy fibers of the dentate gyrus. Before seizure (A1), after seizure (A2). B1, B2, Diagrams of the electrode configuration superimposed on a coronal section of the brain. Local EEG was simultaneously recorded from the striatum (B1) and hippocampus (B2), together with the ipsilateral frontal EEG (B1). The recording sites were histologically verified in the cresyl violet staining sections. C, Ictal EEG activity in three Arx(GCG)7/Y mice (#1, #2, #3). The seizure started (dotted line) simultaneously in the hippocampus, frontal cortex and striatum, followed by diffuse spike bursts. Next, 20-30 Hz spike bursts with waxing and waning appeared in the hippocampus (bar). Then there were continuous bursts of very high-voltage spikes (open bar) in the striatum, which abruptly changed into a long-lasting low-voltage activity with synchronous rhythmic theta waves. All seizures started with limb trembling that progressed to tonic clonic seizures (closed triangle), running fits (arrow) and complete loss of postural control and movement (open arrow). D, Examples of EEG data at the beginning of a seizure in three Arx(GCG)7/Y mice (#1, #2, #3). The first spikes are simultaneously seen in the frontal cortex, hippocampus and striatum. F-cx, frontal cortex;
Hip, hippocampus; Str-l, lower electrode in the striatum; Str-u, upper electrode in the striatum; Str-BP, bipolar in the striatum.

**Figure 5**
**Defects in several aspects of learning and memory in** $Arx^{PL/Y}$ **and** $Arx^{(GCG)7/Y}$ **mice**

**A, B** Latency to enter a darkened chamber in the passive avoidance task. The left set of bars represents results before the mice received electric shocks in the darkened chamber; the right set shows the results after shocks. A significant difference from the $Arx^{X/Y}$ mice is represented by * for $P<0.05$ and *** for $P<0.001$. **C, D**, Accuracy of the win-shift task on the eight-arm radial maze, which we present as the percentage of chosen arms that were baited. The value is given as the mean of three trials. **E, F**, The ratio of the unlit arm choices to lit arm choices in the win-stay task on the eight-arm radial maze. The value is given in the same manner as (C) and (D). The $Arx^{(GCG)7/Y}$ mice and $Arx^{X/Y}$ mice were statistically analyzed from blocks 1 to 4 in (D) and from block 1 to 5 in (F), using the number of the subjects shown in the figures. Mean values are shown on the solid line. The mean values on the broken line were calculated from the number of surviving $Arx^{(GCG)7/Y}$ mice in each block because some of them died during training. The number of subjects surviving throughout the tasks is shown in the parentheses.

**Figure 6**
**Reduction of neurons in each subtype of GABAergic interneurons in the striatum of** $Arx^{PL/Y}$ **and** $Arx^{(GCG)7/Y}$ **mice at P1m**

SST$^+$ interneurons of $Arx^{PL/Y}$ (47.8±4.8%, $P<0.001$, n=3. B, M) and $Arx^{(GCG)7/Y}$ mice (52.4±6.2%, $P<0.001$, n=3. C, M) were about half as many cells within the SST$^+$ interneurons as in $Arx^{X/Y}$ mice (A). The same tendency was seen for the NPY$^+$ (D-F, M) and NOS$^+$ (G-I, M) interneurons. In contrast, the reduction in PV$^+$ interneurons in $Arx^{PL/Y}$ (85.2±4.2%, $P<0.01$, n=3. K, M) and $Arx^{(GCG)7/Y}$ (70.6±7.3%, $P<0.002$, n=3. L, M) mice was small compared with the other subtypes. Values in (M) are normalized to the wild type. Str: striatum. Scale bar: A-L, 500 μm.
Figure 7
Reduction of cholinergic interneurons in the striatum and cholinergic and GABAergic projection neurons in the medial septum and ventral forebrain nuclei in $Arx^{PL/Y}$ and $Arx^{GCG/7/Y}$ mice at P1m

A-C, ChAT$^{+}$ cholinergic interneurons in the striatum. There were about half as many ChAT$^{+}$ interneurons in $Arx^{PL/Y}$ mice (B) as in $Arx^{X/Y}$ mice (A), while no cholinergic interneurons were found in $Arx^{GCG/7/Y}$ mice (C). D-F, ChAT$^{+}$ cholinergic projection neurons in the medial septum (MS) and vertical limbs of the nucleus of the diagonal bands (DBv). The number of neurons in the MS of $Arx^{PL/Y}$ mice was reduced to 53.2±4.8% of that of $Arx^{X/Y}$ mice (P<0.001, n=3, D, E), whereas it was reduced to 23.4±4.0% of that of $Arx^{X/Y}$ mice in $Arx^{GCG/7/Y}$ mice (P<0.001, n=3, F). The number of neurons in the DBv of $Arx^{PL/Y}$ mice was reduced compared with the MS. G-I, GAD67$^{+}$ projection neurons in the MS and DBv. The number of neurons was severely reduced in the MS and DBv of $Arx^{PL/Y}$ mice (H), and no neurons were found in the MS or DBv of $Arx^{GCG/7/Y}$ mice (I), except for GAD67 signals in the most anterior medial MS and DBv (inset of I). J-L, ChAT$^{+}$ cholinergic projection neurons in the magnocellular preoptic nucleus. The numbers of cholinergic projection neurons in $Arx^{PL/Y}$ (K, P) and $Arx^{GCG/7/Y}$ (L, P) mice were 80.1±8.5% (P<0.01, n=3) and 68.4±7.5% (P<0.001, n=3), respectively, of that in $Arx^{X/Y}$ mice (J). M-R, S, Acetylcholinesterase (AchE)$^{+}$ axonal arbors of cholinergic projection neurons in the hippocampus (M-O, S) and cortex (P-R, S). The total length of AchE$^{+}$ fibers per arbitral area in the hippocampus (molecular layer of dentate gyrus) of $Arx^{PL/Y}$ (N) and $Arx^{GCG/7/Y}$ (O) mice was 50.5±5.2% (P<0.01, n=3), and 16.4±6.7% (P<0.001, n=3), respectively, of that in $Arx^{X/Y}$ mice (M). The total length of AchE$^{+}$ fibers per arbitral area in the somatosensory cortex of $Arx^{PL/Y}$ (Q) and $Arx^{GCG/7/Y}$ (R) mice was 65.5±9.8% (P<0.01, n=3), and 15.8±6.6% (P<0.001, n=3), respectively, of that in $Arx^{X/Y}$ mice (P). Values in (S) are normalized to wild type. Str: striatum, MS: medial septum, DBv: vertical limbs of the nucleus of the diagonal band, MCPO: magnocellular preoptic nucleus, DG: dentate gyrus, Cx: cortex. Scale bars: A-C, 500 μm, D-I, 500 μm, J-L, 500 μm, M-O, 100 μm, P-R, 200 μm, M-O, 100 μm, P-R, 50 μm.
Table 1
Seizure induction by bicuculline in \textit{Arx}^{\text{PL/Y}} mice

(A) The number of mice displaying myoclonic jerks and generalized seizures, and of deaths in the \textit{Arx}^{\text{PL/Y}} and \textit{Arx}^{\text{XY}} mice.

(B) The onset of myoclonic jerks, generalized seizures and death from injection in the \textit{Arx}^{\text{PL/Y}} and \textit{Arx}^{\text{XY}} mice.

\begin{tabular}{|l|l|c|c|c|}
\hline
postnatal month old & genotype & mice with myoclonic jerks / total & mice with generalized seizure / total & deaths / total \\
\hline
P1m & \textit{Arx}^{\text{PL/Y}} & 6/6 & 6/6 & 6/6 \\
& \textit{Arx}^{\text{XY}} & 6/6 & 4/6 & 1/6 \\
& \textbf{P} & 1 & 0.4546 & 0.0152* \\
\hline
P3-5m & \textit{Arx}^{\text{PL/Y}} & 8/8 & 8/8 & 8/8 \\
& \textit{Arx}^{\text{XY}} & 7/8 & 3/8 & 2/8 \\
& \textbf{P} & 1 & 0.0256* & 0.0070** \\
\hline
\end{tabular}

\begin{tabular}{|l|l|c|c|c|}
\hline
postnatal month old & genotype & time myoclonic jerks start (min) & time generalized seizure start (min) & time of death (min) \\
\hline
P1m & \textit{Arx}^{\text{PL/Y}} & 2.62±0.60 & 3.58±0.97 & 17.00±9.69 \\
& \textit{Arx}^{\text{XY}} & 5.43±0.81 & 6.97±1.83 & 18.00±0.0 \\
& \textbf{P} & 0.0039** & 0.0105* & \\
\hline
P3-5m & \textit{Arx}^{\text{PL/Y}} & 3.34±0.51 & 4.54±1.62 & 9.00±1.69 \\
& \textit{Arx}^{\text{XY}} & 6.57±4.16 & 9.53±5.17 & 15.25±1.06 \\
& \textbf{P} & 0.0038** & 0.0143* & \\
\hline
\end{tabular}

Values are means ± SD, *P<0.05 ; **P<0.01
Figure 1

A. Introduction of PR and PL mutations

B. Introduction of (GCG)7 mutation

C. PR mutation

D. (GCG)7 mutation

E. ARX

F. Mutant/Wild type (%)

G. WT, PR, PL, (GCG)7

H. WT, PR, PL, (GCG)7
Figure 2
Figure 3
Figure 4
Figure 5

A

B

C

D

E

F

Latency of entry into the darkened chamber (sec)

Arx^XY^:

n=14

Arx^PL/Y^:

n=12

Naive

Experienced

Arx^XY^:

n=15

Arx^X/Y^:

n=15

Arx^(GCG)7/Y^:

Arx^XY^:

Arx^PL/Y^:

Arx^X/Y^:

Naive

Experienced

Accuracy of task

Arx^XY^:

n=7

Arx^PL/Y^:

n=12

3-trial blocks

Arx^XY^:

n=12

Arx^(GCG)7/Y^:

3-trial blocks

Arx^XY^:

n=7

Arx^(GCG)7/Y^:

3-trial blocks

Ratio of unit arms to lit arms

Arx^XY^:

n=11

Arx^PL/Y^:

n=13

3-trial blocks

Arx^XY^:

n=7

Arx^(GCG)7/Y^:

n=4

3-trial blocks
Figure 6
ABBREVIATIONS

AchE  Acetycholinesterase
ARX  aristaless-related homeobox
Arx(GCG)7/Y  ArxY
ArxPR/Y  ArxP355R/Y
ArxPL/Y  ArxP355L/Y
CP  cortical plate
Cx  cortex
ChAT  choline acetyltransferase
DBh  horizontal limbs of the nucleus of the diagonal bands
DBv  vertical limbs of the nucleus of the diagonal bands
DG  dentate gyrus
DL  down layer
EEG  electroencephalographic
ES  embryonic stem
GABA  γ-aminobutyric acid
GAD  glutamate decarboxylase
Gapdh  glyceraldehyde-3-phosphate dehydrogenase
GE  ganglionic eminence
MCPO  magnocellular preoptic nucleus
MGE  medial ganglionic eminence
ML  middle layer
MS  medial septum
NOS  NO synthase
NPY  neuropeptide Y
PFA  paraformaldehyde
PV  parvalbumin
SST  somatostatin
Str  striatum
UL  upper layer
XLAG  X-linked lissencephaly with ambiguous genitalia