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Arx acts as a regional key selector gene in the ventral telencephalon mainly through its transcriptional repression activity

Gaia Colasante [a], Alessandro Sessa [a], Stefania Crispi [b], Raffaele Calogero [c], Ahmed Mansouri [d], Patrick Collombat [e], Vania Broccoli [a,*]

[a] Stem Cell and Neurogenesis Unit, Division of Neuroscience, San Raffaele Scientific Institute, Via Olgettina 58, 20132, Milan, Italy
[b] Gene Expression Core, Human Molecular Genetics laboratory, IGB-CNR, Via Castellino 111, 80131 Naple, Italy
[c] Department of Clinical and Biological Sciences, University of Turin, Turin, Italy
[d] Department of Molecular Cell Biology, Max Planck Institute for Biophysical Chemistry, 37077, Göttingen, Germany
[e] Mouse Molecular Genetics Department, Inserm U636, Faculté des Sciences, 06108, Nice, France

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ABSTRACT

The homeobox-containing gene *Arx* is expressed during ventral telencephalon development and required for correct GABAergic interneuron tangential migration from the ganglionic eminences to the olfactory bulbs, cerebral cortex and striatum. Its human ortholog is associated with a variety of neurological clinical manifestations whose symptoms are compatible with the loss of cortical interneurons and altered basal ganglia-related activities. Herein, we report the identification of a number of genes whose expression is consistently altered in Arx mutant ganglionic eminences. Our analyses revealed a striking ectopic expression in the ganglionic eminences of several of these genes normally at most marginally expressed in the ventral telencephalon. Among them, *Ebf3* was functionally analyzed. Thus, its ectopic expression in ventral telencephalon was found to prevent neuronal tangential migration. Further, we showed that Arx is sufficient to repress *Ebf3* endogenous expression and that its silencing in Arx mutant tissues partially rescues tangential cell movement. Together, these data provide new insights into the molecular pathways regulated by Arx during telencephalon development.

Introduction

In the last decade, outstanding progress has been made in our actual understanding of the molecular mechanisms involved in the patterning of the telencephalon. Hence, complex interactions between a number of extrinsic and intrinsic cellular factors were implicated in the subdivision of the telencephalon into different territories exhibiting distinct cellular features (Wilson and Rubenstein, 2000; Guillemot, 2005; Rash and Grove, 2006; Hébert and Fishell, 2008). For instance, signaling molecules belonging to the FGF, Wnt, Shh and RA families, secreted from distinct signaling centers, act to establish positional information along the anterior neuraxis and thereby create a primitive protomap of the telencephalic primordium (Shimamura and Rubenstein, 1997; Fuccillo et al., 2004; Marklund et al., 2004; Shimogori et al., 2004). These nascent territories express specific sets of transcription factors acting to define definitive anatomical and cellular organizations. Recent studies have revealed how successive cascades of such nuclear factors may regulate these processes: early neuronal commitment is induced by bHLH proneural factors which, in turn, interact with homeoproteins or other nuclear proteins to define the cell fate and organization of each territory (Castro et al., 2006; Gohlke et al., 2008). In particular, in the ventral telencephalon, the Mash1 proneural factor directly promotes the transcription of the *Dlx1* and *2* homeobox genes, which in turn, support GABAergic subtype neuronal differentiation and tangential migration (Cobos et al., 2005a,b; Cobos et al., 2007; Poitras et al., 2007). Concomitantly, Dlx1 and 2 proteins activate the Dlx5, Dlx6 and Arx transcription factors (Zerucha et al., 2000; Colasante et al., 2008). Dlx5 and 6 were found to play a major role in controlling olfactory bulb interneuron commitment and migration (Levi et al., 2003; Long et al., 2003). Conversely, Arx displays multiple functions in ventral telencephalon morphogenesis, in radial and tangential migrations of GABAergic

* Corresponding author. Developmental Neuropathology Laboratory, San Raffaele Scientific Institute, Via Olgettina, 58, 20132 Milan, Italy. Fax: +39 2 26434621.
E-mail address: broccoli.vania@hsr.it (V. Broccoli).

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neuronal progenitors, and in early commitment of cholinergic neurons (Kitamura et al., 2002; Colombo et al., 2007; Friocourt et al., 2008). Notably, mutations in the human ARX ortholog were associated with a wide range of neurological disorders. For instance, missense mutations or expansion of polyalanine tracts were linked to an important number of neuropathological diseases, including idiopathic mental retardation, epilepsy, early infantile epileptic encephalopathy and diskinetic quadriparesis, as well as more complex syndromic conditions, such as West, Proud and Partington syndromes (Strømme et al., 2002; Bienvenu et al., 2002; Kato and Dobyns, 2005; Kato et al., 2005, 2007; Gécz et al., 2006; Guerrini et al., 2007). More severe mutations within the ARX gene resulting in premature termination of the protein, have been reported in X-linked lissencephaly with abnormal genitalia (XLAG) patients (Kitamura et al., 2002; Kato and Dobyns, 2003, 2005). The latter exhibit severe brain malformations, including agenesis of corpus callosum, reduced and fragmented basal ganglia, and severe reduction in cortical GABAergic interneuron numbers associated with their aberrant distribution in the white matter and neocortical subventricular zone (Kitamura et al., 2002; Gécz et al., 2006; Colombo et al., 2007; Okazaki et al., 2008). Interestingly, such phenotypic malformations are also detectable in neonatal Arx mutant mice, although their perinatal death, due to pancreatic complications, has prevented a consistent behavioral analysis (Kitamura et al., 2002; Colombo et al., 2007; Collombat et al., 2003).

Despite its dramatic impact on brain morphogenesis, very little is known concerning the mechanisms underlying Arx pleiotropic activities, as well as its downstream targets. Few research groups have recently initiated a systematic identification of the molecular effectors of key transcription factors acting throughout brain development (Castro et al., 2006; Gohlke et al., 2008). Such pioneer studies have allowed us to gain further insight into the transcriptional machinery controlling brain neurogenesis. Herein, aiming to uncover at least some of the Arx downstream targets, we performed a gene expression profile analysis comparing wild-type (wt) and Arx mutant ventral telencephalic tissues. Strikingly, a fairly small number of genes were found consistently misregulated in the mutant tissues as compared to controls. Specifically, *Ebf3* was identified as a putative Arx target gene, its expression being more than six-time increased in Arx-deficient animals. Subsequent functional analyses indicated that such up-regulation in the basal ganglia was detrimental for tangential migration of GABAergic neuronal progenitors. Further, knock-down experiments targeting Ebf3 in Arx mutant tissues were found to only partially rescue neuronal migration defects following radial routes towards striatal and pallidal mantle zones, but also migration pathways leading to the cortical plate.

Materials and methods

Animals

Arx mutant mice (Collombat et al., 2003) were maintained by backcrossing with C57Bl/6 animals. Timed-pregnant mice were considered embryonic day 0.5 (E0.5) on the morning of the confirmation of the vaginal plug. Genotyping was performed by morphological assessment and confirmed by PCR using a mixture of three primers to identify the wild-type (wt) and the mutant alleles, generating 0.25 and 0.4 kb PCR products, respectively (primer 1, 5′-CGGCTCACTACACTTGTTACCGCTTGGTCC-3′; primer 2, 5′-AGCAGCCCTCTTCCTGGTACTGATTGCTC-3′; and primer 3, 5′-TGCTGCAAGGCGATAAGTTGGGTAACGC-3′) (Collombat et al., 2003). Mice were maintained at the San Raffaele Scientific Institute Institutional mouse facility, and experiments were performed in accordance with experimental protocols approved by local Institutional Animal Care and Use Committees (IACUC).

RNA isolation, processing, and microarray analysis

Three timed-pregnant Arx heterozygous dams crossed with C57Bl/6 males were sacrificed at E14.5, their embryos were harvested and placed into cold PBS. Following brain isolation, meninges and olfactory bulbs were removed, and the ventral telencephalon was separated from the overlying cerebral cortex. The same procedure was repeated for 5 wt and 5 Arx mutant embryos. Total RNA was extracted from the tissues using the Qiagen RNA micro kit (Qiagen, Valencia, CA). cRNAs were generated and hybridized on a total of ten different MOE430v2 Affymetrix DNA chips according to the Affymetrix protocol. The chips were scanned with a specific scanner (Affymetrix) to generate digitized image data files. The data were deposited in the NCBI Gene Expression Omnibus (Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002; 30:207–10) and are accessible through GEO Series.

Microarray data analysis

Microarray quality control and statistical validation were performed using Bioconductor (Gentleman et al., 2004; Sanges et al., 2007). Background correction, normalization, and probe set intensities were obtained by means of GCRMA (Wu and Irizarry, 2004). The number of genes evaluated was reduced by applying an interquartile (IQR) filter followed by an intensity filter to remove the not significant probe sets, i.e. not expressed and those not changing (Huber et al., 2002; Cordero et al., 2007). Principal component analysis (PCA) (Raychaudhuri et al., 2000) was used to investigate the overall behavior of the two experimental conditions.

To assess differential expression, we used an empirical Bayes method (Smyth, 2004) together with a false discovery rate (FDR) correction of the *P*-value (Westfall and Young, 1993). Thus, the list of differentially expressed genes was generated using an FDR ≤ 0.05 together with an absolute $\log_2$(fold-change) threshold of 1 and by selecting those probe sets mapping to unique Entrez Gene identifiers (Maglott et al., 2005).

Functional annotation was performed using Ingenuity knowledge base (www.ingenuity.com), which enables the analysis, and the understanding of the complex biological and chemical systems taking advantage of a manually curate knowledge base.

Hierarchical clustering and PCA analysis were performed using TMEV software (www.tigr.org). Array data were deposited on GEO data base (# GSE12956).

Quantitative real-time PCR and quantification

2 μg of the total RNA extracted from wt and Arx mutant ventral telencephali were reverse transcribed with random hexamers as primers using a Thermoscript RetroTranscriptase (Invitrogen). The oligonucleotides used for the amplification of selected genes are reported in Table S2. qRT-PCRs were carried out in a final volume of 25 μl, containing a concentration of 100 nM of each primer, 1× Syber Green mix (Biorad) and 2 μl of the RT products. Thermal cycling was performed using a Mx3005P QPCR system (Stratagene). Melting curve analysis was performed for each reaction to ensure a single peak and amplicons were visualized after electrophoresis on a 2% agarose gel to ensure the presence of a single PCR product.

The Livak method was applied for quantification (Schmittgen and Livak, 2008). Briefly, the expression of each gene either in wild-type or in mutant samples was normalized to that of the housekeeping gene *β-actin*: to this purpose a $\Delta C_{T,\ WT} = (C_{T,\ gene} - C_{T,\ act})_{WT}$ and a $\Delta C_{T,\ MUT} = (C_{T,\ gene} - C_{T,\ act})_{MUT}$ were calculated for each amplified gene and results were reported as fold change ($2^{-\Delta\Delta CT}$) in gene expression of Arx mutant samples relative to the wild-type, where $-\Delta\Delta C_T = -(\Delta C_{T,\ MUT} - \Delta C_{T,\ WT})$.