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Research report

Knockout of sodium pump α3 subunit gene (*Atp1a3*$^{-/-}$) results in perinatal seizure and defective respiratory rhythm generation

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ABSTRACT

ATP1A3 encodes a neuron-specific human α3 subunit isoform of the sodium pump that plays an important role in neuronal excitability. Point and deletion mutations in *ATP1A3* have been recognized in diverse neurological disorders. Three *ATP1A3* disorders, alternating hemiplegia of childhood (AHC); apnea; and severe infantile epileptic encephalopathy often appear shortly after birth. To gain insight into the pathophysiology of these disorders and to understand the functional roles of the sodium pump α3 subunit in the brain *in vivo* during this period of development, we examined the phenotype of *Atp1a3* knockout homozygous mouse fetuses (*Atp1a3*$^{-/-}$). We focused on fetuses just before birth because at birth, about half of them showed severe seizure, and none could continue effective breathing and died soon after birth, without any gross anatomical anomalies. We examined c-Fos expression in the brains of *Atp1a3*$^{-/-}$ and found a significantly increased number of c-Fos-expressing cells in various regions of the brains, with unique distribution in the cerebellum, when compared with wild-type littermates (*Atp1a3*$^{+/+}$). We also measured contents of monoamine neurotransmitters in the brains and found higher contents, especially of dopamine and noradrenaline, in the brains of *Atp1a3*$^{-/-}$ compared with those of *Atp1a3*$^{+/+}$. In addition, we found various abnormal respiratory rhythms produced in the brainstem of *Atp1a3*$^{-/-}$. These results suggest that *Atp1a3* plays a critical role in neural function during development and at birth.

1. Introduction

Over the past several years, heterozygous mutations of *ATP1A3* have been increasingly reported in various neurological disorders. *ATP1A3* encodes the human α3 subunit of the sodium pump. The sodium pump (or Na,K-ATPase) is the enzyme responsible for maintaining Na^+ and K^+ gradients across the animal cell membrane. The electrochemical gradient is of fundamental importance for the transport of various nutrients and ions, regulation of cellular osmolality and cell volume, and electrical excitability of nerves and muscles. The sodium pump is composed of α and β subunits. The α subunit contains the binding sites for cations (Na^+ and K^+) and the catalytic site of ATP hydrolysis (Lingrel and Kuntzweiler, 1994). There are four α isoforms (α1, α2, α3, and α4) and three β isoforms (β1, β2, and β3) in mammals with different expression patterns. Most neurons express α1 and/or α3 with β1 subunits, whereas glial cells express α2 with β2 and/or β3 subunits in the adult human and rodent brains. Heterozygous mutations in *ATP1A3* have been identified in distinct but related human neurological disorders such as rapid–onset dystonia parkinsonism (RDP or DYT12, MIM 128235) (Brashear et al., 2007; de Carvalho Aguiar et al., 2004), alternating hemiplegia of childhood (AHC, MIM614820) (Heinzen et al., 2012; Rosewich et al., 2012), and cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss syndrome (CAPOS, MIM 601338) (for review, Brashear et al.,

Abbreviations: 3-MT, 3-methocytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; AHC, alternating hemiplegia of childhood; *ATP1A3* or *Atp1a3*, gene for sodium pump α3 subunit of human or mouse, respectively; *Atp1a3*, homozygous knockout mice of *Atp1a3*; *Atp1a3*$^{+/-}$, heterozygous knockout mice of *Atp1a3*; *Atp1a3*$^{+/+}$, wild-type mice; CAPOS, cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss; c pFRG, caudal parafacial respiratory group; DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxypenylacetic acid; GABA, γ-aminobutyric acid; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; NA, norepinephrine; NMET, normetanephrine; pFRG, parafacial respiratory group; preBötC, pre-Bötzinger complex inspiratory group; RDP, rapid-onset dystonia parkinsonism; r pFRG, rostral parafacial respiratory group.

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2008; Demos et al., 2014). Moreover, *ATP1A3* mutation is associated with severe infantile epileptic encephalopathy, which is the most severe phenotype with seizures within the early days of life, and with poor survival or episodic apnea and severe developmental disability (Paciorkowski et al., 2015; Sasaki et al., 2014). With regard to epileptic seizure, a few patients with RDP and more than half of the patients with AHC show episodes of seizures (Bøttger et al., 2012). Incurable neonatal seizures, early-life epilepsy, and *status epilepticus* have been reported in clinically defined AHC patients (Ishii et al., 2013; Rosewich et al., 2012, 2014; Saito et al., 2010). However, the primary focus of seizures and the sites of neural hyperexcitation involved in paroxysmal epileptic attacks have not been identified yet.

We previously developed the *Atp1a3* knockout mouse to understand the role of the α3 isoform of the sodium pump in brain and analyzed its suitability as a pathophysiological animal model (Ikeda et al., 2013; Sugimoto et al., 2014). We reported that heterozygous *Atp1a3* knockout mice (*Atp1a3*$^{+/-}$) showed enhanced inhibitory neurotransmission in the cerebellum (Ikeda et al., 2013) and exhibited motor deficits following chronic restraint stress (Sugimoto et al., 2014). In addition, we briefly mentioned in Ikeda et al. (2013) that homozygous knockout newborns (*Atp1a3*$^{-/-}$) died just after birth due to complete lack of breathing movements. So far, no patients harboring homozygous mutations of *ATP1A3* have been reported, probably because such mutations are also lethal in humans during the embryonic or neonatal period.

We expected that further analyses of *Atp1a3*$^{-/-}$ would uncover the fundamental roles of the sodium pump α3 subunit during perinatal period because the phenotype manifested by complete absence of the gene reflects the extreme necessity of the gene *in vivo*. Analyses may also provide hints of affected brain regions in patients with *ATP1A3* mutations and explain some aspects of the pathophysiology of neurological disorders such as seizure/epilepsy and dystonia, although the latter is observed in older patients. For these purposes, we revisited how and why *Atp1a3*$^{-/-}$ died just after birth. With careful observation, we found that about half of the *Atp1a3*$^{-/-}$ showed seizures that hindered effective breathing, and the other half showed no body movement or breathing movement effort. Because of the relatively high frequency of the seizure–occurrence phenotype in *Atp1a3*$^{-/-}$, we histologically analyzed c-Fos expression in the brain to identify the hyper-excited brain regions during the perinatal period when formation of the functional neural network has started. Next, we measured the contents of monoamine neurotransmitters in the brain because much experimental, clinical, and genetic evidence has strongly suggested that monoamines play an important role in regulating epileptogenesis and seizure susceptibility (Svob Strac et al., 2016). Also, an imbalance in dopamine (DA) signaling was reported in the dystonic striatum (Augood et al., 2002). Lastly, we examined formation of the functional neural network by monitoring respiratory neural network activity in *Atp1a3*$^{-/-}$ using an electrophysiological approach. We chose this activity for the analyses because the respiratory neural network is established during the embryonic stage to adequately generate involuntary rhythmic neural activity immediately after birth.

2. Results

2.1. Expression of Atp1a3 during the perinatal period

Information on the *Atp1a3* expression pattern is fundamental to understanding *Atp1a3* function in the brain and to gain insight into the pathophysiology of neurological disorders caused by mutation of its orthologous gene *ATP1A3* in humans. Bøttger et al. (2011) described the comprehensive distribution of the α3 protein in neurons of the adult mouse brain using immunohistochemistry. We previously mapped *Atp1a3* mRNA expression in the whole brain of young adult mice at postnatal day (P) 38 by *in situ* hybridization (Sugimoto et al., 2014). Orlowski and Lingrel (1988) reported the expression of α3 subunit mRNA in rat neonatal brain using northern blotting analysis. Herrera et al. (1994) examined *Atp1a3* expression in sections of embryonic day (E)9.5/E10.5 embryos and in limited brain regions of E15.5/E16.5 embryos. The Allen Brain Atlas shows the expression of *Atp1a3* in the whole brain at the age of P56 (http://mouse.brain-map.org/gene/show/87535) but has no data for its expression in the developing brain in the perinatal stage (see http://developingmouse.brain-map.org). Therefore, we first examined mRNA distribution of *Atp1a3* by *in situ* hybridization in the wild-type E18.5-P0 mouse whole brain.

As shown in Fig. 1, *Atp1a3* was expressed in almost all regions of the P0 brain containing neurons: the cerebral cortex, limbic system, basal ganglia, diencephalon, midbrain, pons, medulla oblongata, cerebellum, and in the spinal cord. In the rostral forebrain, *Atp1a3* was expressed in several layers of the main olfactory bulb and the accessory olfactory bulb (Fig. 1*A, B*). *Atp1a3* was also expressed throughout the cerebral neocortex (Fig. 1*B–M*). Strong positive signals were observed in the subplate zone (white arrow in Fig. 1*F*) of the neocortex including the cingulate, motor, and sensory cortexes throughout the sections. The subplate zone is a transient cortical structure and plays an important role in the early establishment and maturation of thalamocortical connections and in the development of inhibitory cortical circuits in sensory areas (reviewed in Hoerder-Suabedissen and Molnár, 2015). In contrast, the subventricular zone showed weak signals for *Atp1a3* (blue arrow in Fig. 1*F*). The piriform cortex, one of the olfactory areas and also known as the primary olfactory cortex or the paleocortex, showed strong positive signals throughout the retrobulbar region rostrally to the entorhinal cortex caudally (Fig. 1*E–J*). Like the piriform cortex, the olfactory tubercle and the diagonal band, both also designated as the primary olfactory cortex, were positive for *Atp1a3* expression (Fig. 1*D, F*). As for the limbic system, clear *Atp1a3* signals were evident in the hippocampal formation (Fig. 1*H–K*) and amygdaloid complex, which is a prominent structure lying in the temporal lobe and comprises several subdivisions (Fig. 1*H–J*). As for the basal ganglia, such as the accumbens nucleus (the main component of the ventral striatum), caudate nucleus and putamen, globus pallidus, and subthalamic nucleus (which is one of the ventral thalamic nuclei in Fig. 1*J*), all were positive for *Atp1a3* (Fig. 1*E–J*). In the diencephalon, various thalamic nuclei (such as the geniculate nucleus in Fig. 1*K*) and hypothalamic nuclei, such as the ventromedial hypothalamic nucleus and the arcuate nucleus (Fig. 1*I–K*), were positive for *Atp1a3*. Other diencephalon structures that are components of the epithalamus, such as the habenular complex and pineal gland, were also positive for *Atp1a3* (Fig. 1*I, J*).

The major structures of the midbrain, such as the superior colliculus, periaqueductal gray, substantia nigra, red nucleus, and inferior colliculus were all positive for *Atp1a3* (Fig. 1*L–Q*, the inferior colliculus was observed until Fig. 1*U* because of angularly tilted sections). The midbrain reticular formation and the oculomotor nucleus were positive for *Atp1a3* (Fig. 1*N*). In the pons, the pontine nucleus, pontine reticular nucleus, median raphe, nucleus trapezoid body, locus coeruleus, and parabrachial nucleus were positive for *Atp1a3* (Fig. 1*N–Q*). The trigeminal motor nucleus and superior olive were strongly positive for *Atp1a3*. In the medulla oblongata, cranial nuclei (such as the facial, cochlear, vestibular, vagus, and hypoglossal nuclei), inferior olive, reticular nucleus, nucleus of solitary tract, and the dorsal funiculus (fasciculus gracilis and fasciculus cuneatus) showed positive signals for *Atp1a3* (Fig. 1*P–X*). In the developing cerebellum, Purkinje cell progenitors in the simple lobule and hemisphere and medial cerebellar nuclei were strongly positive for *Atp1a3*, as were other cerebellar nuclei