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Pathogenesis of severe ataxia and tremor without the typical signs of neurodegeneration

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ABSTRACT

Neurological diseases are especially devastating when they involve neurodegeneration. Neuronal destruction is widespread in cognitive disorders such as Alzheimer's and regionally localized in motor disorders such as Parkinson's, Huntington's, and ataxia. But, surprisingly, the onset and progression of these diseases can occur without neurodegeneration. To understand the origins of diseases that do not have an obvious neuropathology, we tested how loss of CAR8, a regulator of IP3R1-mediated Ca^{2+}-signaling, influences cerebellar circuit formation and neural function as movement deteriorates. We found that faulty molecular patterning, which shapes functional circuits called zones, leads to alterations in cerebellar wiring and Purkinje cell activity, but not to degeneration. Rescuing Purkinje cell function improved movement and reducing their Ca^{2+} influx eliminated ectopic zones. Our findings in *Car8*^{wdl} mutant mice unveil a pathophysiological mechanism that may operate broadly to impact motor and non-motor conditions that do not involve degeneration.

1. Introduction

The severity of neurological disease increases with neurodegeneration. In Alzheimer's disease, cognition declines with widespread neuronal destruction and in Parkinson's, Huntington's, and ataxia movement rapidly deteriorates with the onset of neurodegeneration (Gennarino et al., 2015). However, neurodegeneration may not be a prerequisite for such dysfunction. Here, we used the cerebellum as a model to uncover how an intact circuit can still impact disease outcome.

The cerebellum is involved in a number of motor disorders including ataxia, dystonia, and tremor (Louis et al., 2011; Orr, 2012; Wilson and Hess, 2013). Purkinje cells are the presumed source of these disorders, and they often degenerate (Unno et al., 2012; Orr, 2012; Prudente et al., 2013; Louis, 2014). Yet in some diseases, movement is obstructed before Purkinje cells degenerate (Shakkottai et al., 2011). In other cases, motor problems start early in life before circuits mature, without leading to massive degeneration (Pandolfo, 2008). This raises a critical question; what features of Purkinje cell wiring influence motor disease when basic circuit anatomy persists?

To address this problem, we first needed to identify an appropriate model. We found that the spontaneous mutant mouse, *waddles*, may be ideal for several reasons. W*addles* (*wdl*) mice contain a deletion in exon 8 of the carbonic anhydrase 8 gene (*Car8*), creating a null allele with no protein (Jiao et al., 2005). In the brain, CAR8 protein is expressed predominantly in Purkinje cells. Its expression is initiated during embryogenesis and maintained into adulthood (Kato, 1990; Taniuchi et al., 2002). CAR8 belongs to a family of zinc metalloenzymes that catalyze the reversible hydration of CO_2 (Tripp et al., 2001), although it lacks the catalytic domain that would make it an active carbonic anhydrase (Kato, 1990). It does, however, bind to inositol 1,4,5-triphosphate receptor type 1 (IP_3R1), with the effect of decreasing the affinity of IP_3 for its receptor (Hirota et al., 2003). *Car8*^{wdl} mice have ataxia and appendicular dystonia, with cerebellar microcircuit abnormalities (Hirasawa et al., 2007) occurring without gross anatomical defects (Jiao et al., 2005). In humans, mutations in the homologous gene, *CA8*, also cause ataxia and a predisposition for quadrupedal locomotion (Turkmen et al., 2009). *CA8*, with at least three other ataxia/tremor causing genes, define this heterogeneous condition called CAMRQ (Ali et al., 2012). Interestingly, *ITPR1* mutations cause SCA15 spinocerebellar ataxia, which can also involve tremor (Van de Leemput et al., 2007). The pathogenic roles of *Car8* suggested to us that *Car8*^{wdl} mice could be useful for testing how motor diseases arise without neurodegeneration.

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We tested how three major features of circuit connectivity impact ataxia pathogenesis and the progression of movement-associated tremor: zonal patterning, Purkinje cell firing, and Purkinje cell neurodegeneration. Two possibilities were that in *Car8*^{wdl}, ataxia and tremor are initiated by developmental defects, with motor deficits emerging consequently either because of neuronal misfiring or degeneration. To differentiate between these possibilities, we combined the *Car8*^{wdl} model with molecular zone analyses, neural tracing, *in vivo* electrophysiology, pharmacological manipulations, and behavioral paradigms. We uncovered an unexpected role for cerebellar wiring during ataxia and tremor pathogenesis that does not involve degeneration or cell loss.

2. Materials and methods

2.1. Animals

Car8^{wdl} mutants (*wdl*/*wdl*) and C57BLKS/J controls were purchased from The Jackson Laboratory (Bar Harbor, ME) and then maintained in our animal colony under an approved IACUC animal protocol according to the institutional guidelines at Baylor College of Medicine.

2.2. Mouse perfusion and tissue procedures

The mice were perfused with 4% paraformaldehyde and the tissue cut on a cryostat. Immunohistochemistry, in situ hybridization, and neural tracing were carried out as described previously (White et al., 2014; see Supplemental Information).

2.3. Western blotting

For each blot, mutant and control cerebella from P30 mice were rapidly dissected and then placed in RIPA buffer containing protease inhibitors before homogenization. We then performed standard SDS polyacrylamide gel electrophoresis (Sillitoe et al., 2003).

2.4. Drug treatment

Chlorzoxazone (CHZ; Sigma, St. Louis, MO, USA) was administered orally by adding the drug to the drinking water to make a 15 mM solution (Alvina and Khodakhah, 2010) and nimodipine (Sigma, St. Louis, MO, USA) was given subcutaneously at a dosage of 5 ml/kg.

2.5. Behavioral analysis

Rotarod performance was quantified by recording the latency to fall or to rotate 3 consecutive times on an accelerating rod (White et al., 2014), and tremor amplitude and frequency were analyzed on a single trial with a Tremor Monitor (San Diego Instruments). Between-group differences were statistically evaluated by Student's t-test. Between-trial differences were statistically evaluated with repeated measures ANOVA.

2.6. In vivo electrophysiology

Mice were anesthetized with Ketamine/Dexmedetomidine (75 mg/kg and 0.5 mg/kg respectively) and maintained with ~0.15%–0.25% isoflurane (White et al., 2014). Single unit recordings were attained from Purkinje cells with 5–8 MΩ tungsten electrodes (Thomas Recording, Germany) and digitized into Spike2 (CED, England). Spike frequency, ISI CV, CV2, rhythm index, and oscillation frequency were computed and reported as mean ± standard error of the mean (SEM). Firing frequency is defined as the number of spikes over a predetermined period of a recording, CV is calculated as the ratio of the standard deviation (SD) of ISIs to the mean ISI of a given cell, CV2 ($=2|ISI_{n+1} - ISI_n|/(ISI_{n+1} + ISI_n)$) measures firing pattern variability within a short period of two interspike intervals (Holt et al., 1996), and rhythm index is a measure of the strength of oscillating patterns within a given period. Oscillation frequency was calculated as the inverse of the time lag of the first peak ($1/t_1$) on autocorrelograms of simple spike activity. Please refer to the Supplemental Information for more details on the electrophysiology methods and the types of spike analyses that were conducted. Statistical analyses were performed with unpaired, two-tailed Student's t-tests. Significance is indicated in the graphs for $p < 0.05$, $p < 0.01$ or $p < 0.001$ with *, **, and ***, respectively.

3. Results

3.1. Movement and learning deteriorate over time and a severe tremor develops in *Car8*^{wdl}

Car8 mRNA and protein are highly localized to cerebellar Purkinje cells (Taniuchi et al., 2002; Lakkis et al., 1997a; Lakkis et al., 1997b) (Fig. 1; n = 6 mutants and 9 controls Figs. S1, S2, S3). We therefore tested the extent to which the loss of CAR8 causes motor defects in juvenile, adult, and aged mice. Motor impairments were obvious between the second to third postnatal week when the *Car8*^{wdl} mice are learning to make independent movements (Movies S1, S2). We used the rotarod assay to test whether motor performance declines from juveniles at P30 through several adult ages and into aging. Indeed, as the mutant mice aged, their motor performance worsened significantly whereas the aged control mice were able to perform to a similar level as younger controls (Fig. 2A). Notably, we found that, at all ages, *Car8*^{wdl} mutants performed poorly compared to their age-matched controls when we examined the performance trajectory to the fourth day of the rotarod paradigm (P30: control = 277.545 ± 12.524 s, *Car8*^{wdl} mutants = 134.969 ± 17.559 s, $p = 2.577 \times 10^{-30}$; P90: control = 285.042 ± 10.117 s, *Car8*^{wdl} mutants = 41.381 ± 11.207 s, $p = 1.244 \times 10^{-30}$; P360: control = 273.267 ± 5.066 s, *Car8*^{wdl} mutants = 15.519 ± 5.912 s, $p = 3.409 \times 10^{-27}$; Fig. 2A,B). Next, we wanted to test whether motor learning is defective in the *Car8*^{wdl} mouse. By comparing P30, P90, and P360 mice over multiple days and trials, we found that, in *Car8*^{wdl} mice, motor learning on the rotarod improves significantly at P30 but not over the 12 trials at P90 or P360 (P30 *Car8*^{wdl}: $F_{(11, 110)} = 3.437$, $p = 0.0004$; P90 *Car8*^{wdl}: $F_{(11, 66)} = 1.681$, $p = 0.0973$; P360 *Car8*^{wdl}: $F_{(11, 88)} = 0.4660$, $p = 0.9194$; Repeated measures, one-way ANOVA; Fig. 2). In addition to the disturbance in locomotion and motor learning, we observed that *Car8*^{wdl} mice shake uncontrollably (Movies S2, S3). We wondered whether this behavior might be indicative of a cerebellar-derived tremor as recent work indicates a strong dependence of tremor on the Purkinje cell circuit (Louis, 2014). To test this possibility, we used a Tremor Monitor to quantitatively measure the frequency and power of rhythmic, shaking movements. Typically, the peak of cerebellar action tremor occurs at a frequency between 4 and 14 Hz (Miwa, 2007; Park et al., 2010; Handforth, 2012). The tremor in control and *Car8*^{wdl} mice both occurred between 4 and 14 Hz. However, the peak tremor of *Car8*^{wdl} mice of all ages examined was of a lower frequency than the expected "physiological tremor" in controls (control: 12.487 ± 0.260 Hz; *Car8*^{wdl}: 9.237 ± 0.178; $p = 7.226 \times 10^{-19}$; Fig. 2) and they have a much higher power (control: $2.13 \times 10^{-4} \pm 1.567 \times 10^{-5}$; *Car8*^{wdl}: $7.52 \times 10^{-4} \pm 5.73 \times 10^{-5}$; $p = 4.233 \times 10^{-15}$; Fig. 2C). We then tested whether the tremor became more pronounced over time, and indeed, by comparing P30, P90, and P360 *Car8*^{wdl} mutants, we found that tremor amplitude increases, suggesting that tremor severity was sensitive to age (Fig. 2; P30 = $1.46 \times 10^{-4} \pm 3.319 \times 10^{-5}$; P90 = $6.42 \times 10^{-4} \pm 7.266 \times 10^{-5}$, P180 = $8.15 \times 10^{-4} \pm 9.891 \times 10^{-5}$; P360 = $1.41 \times 10^{-3} \pm 1.54 \times 10^{-4}$; $F_{(3, 76)} = 26.75$, $p < 0.0001$; Fig. 2C,D). But, interestingly, as tremor amplitude increases with age in *Car8*^{wdl} mutants, frequency decreases (Fig. 2; P30 = 10.5 ± 0.492 Hz; P90 = 9.48 ± 0.356 Hz, P180 = 9.05 ± 0.336 Hz; P360 = 8.176 ± 0.312 Hz; $F_{(3, 74)} = 5.639$, $p = 0.0016$; Fig. 2C) in a manner that is strikingly reminiscent of human essential tremor patients