Neurobehavioral deficits in mice lacking the erythrocyte membrane cytoskeletal protein 4.1

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The erythrocyte membrane cytoskeletal protein 4.1 (4.1R) is a structural protein that confers stability and flexibility to erythrocytes via interactions with the cytoskeletal proteins spectrin and F-actin and with the band 3 and glycophorin C membrane proteins. Mutations in 4.1R can cause hereditary elliptocytosis, a disease characterized by a loss of the normal discoid morphology of erythrocytes, resulting in hemolytic anemia [1]. Different isoforms of the 4.1 protein have been identified in a wide variety of nonerythroid tissues by immunological methods [2–5]. The variation in molecular weight of these different 4.1 isoforms, which range from 30 to 210 kDa [6], has been attributed to complex alternative splicing of the 4.1R gene [7]. We recently identified two new 4.1 genes: one is generally expressed throughout the body (4.1G) [8] and the other is expressed in central and peripheral neurons (4.1N) [9]. Here, we examined 4.1R expression by *in situ* hybridization analysis and found that 4.1R was selectively expressed in hematopoietic tissues and in specific neuronal populations. In the brain, high levels of 4.1R were discretely localized to granule cells in the cerebellum and dentate gyrus. We generated mice that lacked 4.1R expression; these mice had deficits in movement, coordination, balance and learning, in addition to the predicted hematological abnormalities. The neurobehavioral findings are consistent with the distribution of 4.1R in the brain, suggesting that 4.1R performs specific functions in the central nervous system.

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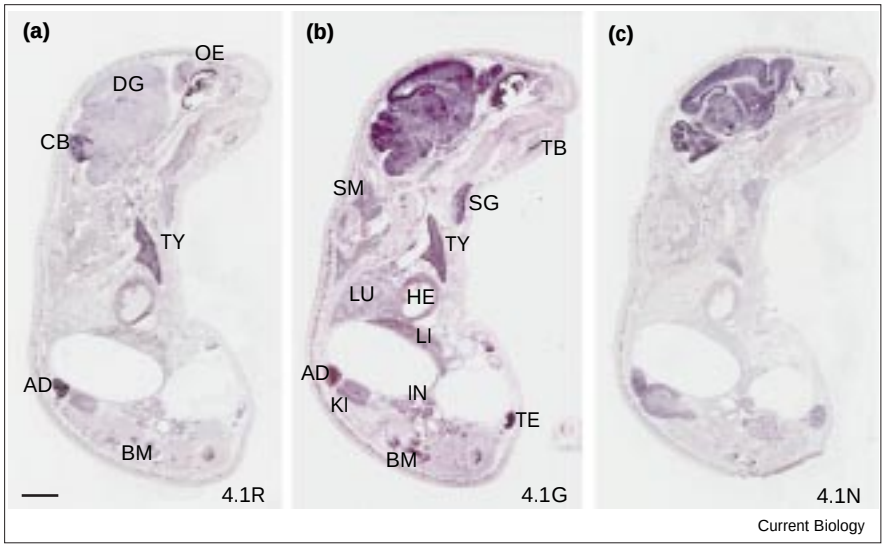
Results and discussion

Isoform-specific probes were used for *in situ* hybridization experiments to determine the specific expression patterns of the different 4.1 genes. High levels of 4.1G mRNAs were detected in the nervous, respiratory, gastrointestinal, hematopoietic, skeletal muscle, salivary and reproductive systems of newborn mice (Figure 1b); 4.1N mRNAs were localized to virtually all central and peripheral neurons (Figure 1c). Interestingly, the expression of 4.1R was predominantly restricted to hematopoietic and nervous tissues. In newborn mice, 4.1R mRNAs were prominent in bone marrow, spleen, liver, thymus and neuronal populations of the cerebellum, dentate gyrus, olfactory system, adrenal medulla and retina (Figures 1a,2a–d and data not shown). In the adult rat brain, however, high levels of 4.1R were localized discretely in the granule cells of the cerebellum and dentate gyrus (Figure 2a–d).

To investigate the functions of 4.1R, we generated mice that had a targeted deletion of exons 2–4 of the 4.1R gene [10]. Western blot analysis of erythrocyte preparations devoid of hemoglobin purified from wild-type ($4.1R^{+/+}$), heterozygous ($4.1R^{+/-}$), and homozygous ($4.1R^{-/-}$) mice confirmed the absence of 4.1R expression in $4.1R^{-/-}$ erythrocytes [10]. *In situ* hybridization also confirmed the lack of expression of 4.1R mRNA in the brains of $4.1R^{-/-}$ animals (Figure 2e–h). Hematologic evaluation revealed that the $4.1R^{-/-}$ mice suffered from aberrant erythrocyte morphology and hemolytic anemia [10]. No statistically significant differences were found between $4.1R^{+/+}$, $4.1R^{+/-}$ and $4.1R^{-/-}$ mice in body mass or length, appetite, primary olfactory abilities (monitored as the latency to sniff a cookie), visual or tactile reflexes as assessed by contact and visual placement, or anxiety levels as evaluated in an open field test and elevated plus maze (described in Supplementary material published with this paper on the internet).

To assess cerebellar function, we monitored movement, balance and coordination. The $4.1R^{+/-}$ and $4.1R^{-/-}$ mice took more than twice as long as $4.1R^{+/+}$ animals to walk one body length (Figure 3a) and, when suspended by their forelimbs on a wire, these mice fell more than twice as quickly as $4.1R^{+/+}$ mice (Figure 3b). In addition, the $4.1R^{+/-}$ and $4.1R^{-/-}$ mice fell when attempting to cross a bridge that was 2 cm wide, but not a bridge that was 4 cm wide (Figure 3c,d). In elevated plus maze and open field

Figure 1

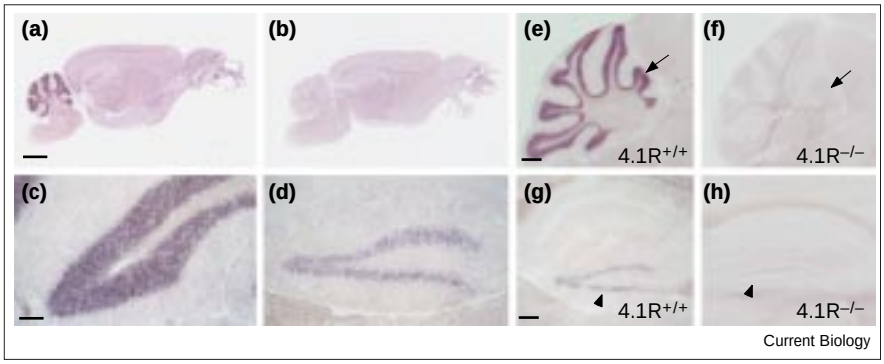


Differential localization of (a) 4.1R, (b) 4.1G, and (c) 4.1N expression in newborn mice. *In situ* hybridization using isoform-specific 4.1 complementary RNA probes was used to identify the specific tissue distributions of the three 4.1 mRNAs [14]. (a) The 4.1R mRNAs were detected in cerebellum (CB), dentate gyrus (DG) of the hippocampus, olfactory epithelium (OE), thymus (TY), adrenal medulla (AD), and bone marrow (BM). (b) The 4.1G transcripts were expressed generally throughout the body, including tooth buds (TB), submandibular gland (SG), thymus (TY), skeletal muscle (SM), lung (LU), heart (HE), liver (LI), adrenal medulla (AD), kidney (KI), intestines (IN), testis (TE), and bone marrow (BM). In the brain, 4.1G mRNA was found in glia and in select neuronal populations. (c) The 4.1N mRNAs were readily detected in all neurons of the brain, excluding several thalamic nuclei. The bar in (a) corresponds to 2.7 mm.

tests [11], which measure animal activity and anxiety levels, $4.1R^{+/-}$ and $4.1R^{-/-}$ mice were indistinguishable from $4.1R^{+/+}$ animals except that the frequency of rearing and the time spent grooming themselves (autogrooming) were significantly reduced (Figure 3e,f). The defects in fine motor skills needed for rearing and autogrooming, and the difficulties with balance and coordination needed for performance on a narrow bridge, were consistent with cerebellar impairment, presumably reflecting the loss of 4.1R in cerebellar granule cells. Interestingly, both $4.1R^{+/-}$ and $4.1R^{-/-}$ animals were impaired in these studies, indicating that both alleles of 4.1R are required for normal neuronal function.

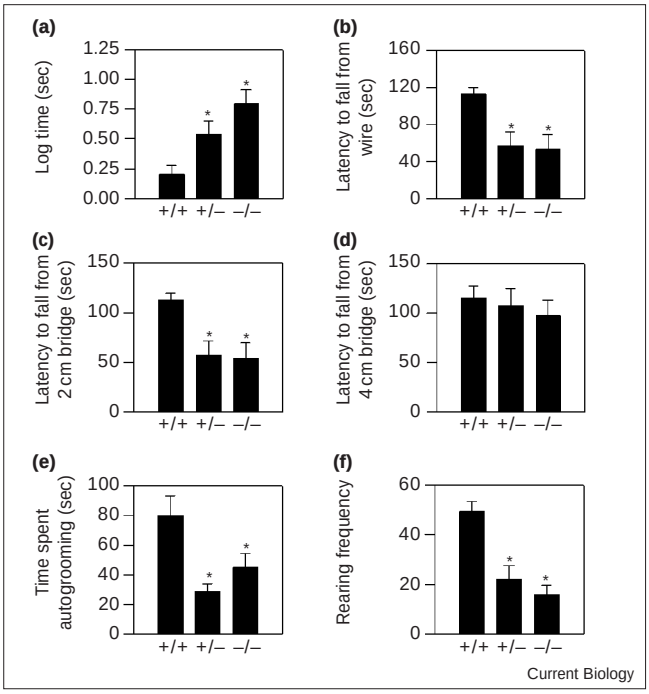
The localization of 4.1R in the dentate gyrus, a region of the brain implicated in learning and memory, prompted us to evaluate spatial memory in $4.1R^{-/-}$ mice using a radial arm maze and a spontaneous alternation task [12]. Both of these are simple maze tests, the radial arm maze dealing with spatial memory and the spontaneous alternation task focusing on simple reference memory. Consistent with the long latencies to initiate walking, $4.1R^{-/-}$ mice completed fewer trials than $4.1R^{+/+}$ animals (Table 1), thereby precluding a rigorous analysis of their learning skills in these systems. Long latencies to walk, with some animals 'freezing', along with some alternations in rearing and autogrooming raise the possibility of motor and/or emotional defects. The task was simplified into a T-maze configuration, creating a behavioral assay of reference memory [13]. In contrast to the spontaneous alternation and radial arm performance, both $4.1R^{-/-}$ and $4.1R^{+/+}$ mice completed all trials in the T-maze, although

Figure 2



In situ hybridization of 4.1R in adult brain. (a–d) Analysis of 4.1R expression in adult rat brain. (a) At low power, 4.1R mRNA was readily visualized in the granule cells of the cerebellum and dentate gyrus. (b) No signal was detected when a serial section was incubated with the control 4.1R sense probe. (c,d) High-power images confirm the exclusive localization of 4.1R mRNA in granule cells of the (c) cerebellum and (d) dentate gyrus. For (a,b), the bar in (a) corresponds to 1.60 mm; for (c,d), the bar in (c) corresponds to 160 μm. (e–h) Analysis of 4.1R expression in adult brains of $4.1R^{+/+}$ and $4.1R^{-/-}$ mice. The distribution of 4.1R mRNA detected in (e,g) $4.1R^{+/+}$ mice is completely absent in (f,h) $4.1R^{-/-}$ mice. The locations of cerebellar granule cells are indicated by arrows, and granule cells of the dentate gyrus are marked with arrowheads. For (e,f), the bar in (e) corresponds to 0.4 mm; for (g,h), the bar in (g) corresponds to 0.25 mm.

Figure 3



The $4.1R^{+/-}$ and $4.1R^{-/-}$ mice exhibit deficits in movement, balance, coordination and spatial learning. Movement, balance and coordination tests were conducted on 12 female mice of each genotype and were scored blindly. Statistically significant differences ($p < 0.05$) are indicated by an asterisk. Error bars represent standard errors of the mean. **(a)** The length of time taken for the mice to walk one body length was measured and plotted as time on a log scale. The $4.1R^{+/-}$ and $4.1R^{-/-}$ mice took significantly longer than $4.1R^{+/+}$ mice to walk this distance. **(b)** Following suspension of mice by their forelimbs from a wire, the latency to fall from the wire (in sec) was recorded, with a cutoff time of 90 sec; both $4.1R^{+/-}$ and $4.1R^{-/-}$ mice consistently fell from the wire. **(c,d)** The latency to fall (in sec) from (c) a 2 cm and (d) a 4 cm wooden bridge was recorded; animals that did not fall received a 'time-out' score of 120 sec. Although $4.1R^{+/-}$ and $4.1R^{-/-}$ mice consistently fell from the 2 cm bridge, no statistically significant differences were detected between the animals when the 4 cm bridge was used. **(e,f)** In open field testing, $4.1R^{+/-}$ and $4.1R^{-/-}$ mice spent significantly less time engaged in (e) autogrooming and (f) rearing compared with $4.1R^{+/+}$ mice. Similar results were obtained when autogrooming and rearing were monitored using the elevated plus maze (data not shown).

the $4.1R^{-/-}$ mice required significantly more trials than wild-type mice to learn the task (Table 1; successful learning was operationally defined as making four consecutive correct choices). Latencies to make choices did not differ between the genotypes, indicating that learning is not confounded in this paradigm by the locomotor difficulties. Taken together, these results are consistent with a hippocampal-dependent deficit in spatial learning and memory. As T-maze learning can be hippocampal independent, however, no strong conclusions can be drawn regarding a link between the learning deficit and hippocampal 4.1R expression.

Table 1

Learning performance of $4.1R^{+/+}$ and $4.1R^{-/-}$ mice.

	$4.1R^{+/+}$	$4.1R^{-/-}$
Radial arm maze		
Number of mice that completed trials	20	8*
Percentage reaching criterion	80	50
Spontaneous alternation		
Number of mice that completed trials	23	14
Number of arm visits	7.2 ± 1.0	2.74 ± 0.61
T-maze		
Number of mice that completed trials	20	20
Days to reach criterion	9.1	13.6*
Latency to make choice	13.3 ± 2.9	14.7 ± 3.3

Simple and spatial working memory were assessed in mice using spontaneous alternation [12], 12-arm radial maze [12], and T-maze [13] tests. Statistically significant differences ($p < 0.05$) are indicated by an asterisk. In radial arm maze testing, fewer $4.1R^{-/-}$ mice reached criterion performance. These results were not statistically significant because only 6 out of 12 $4.1R^{-/-}$ mice were able to complete the task. Analogous findings were obtained from spontaneous alternation studies. Mice were then evaluated in a simple T-maze task [13], which required less overall activity; compared with $4.1R^{+/+}$ mice, $4.1R^{-/-}$ animals took significantly longer to reach criterion performance.

The expression of erythroid 4.1 in such a discrete subset of neurons in the brain is striking. This pattern contrasts with the extensive distribution of most cytoskeletal proteins, including 4.1G and 4.1N, in the brain. Presumably, the neurons containing 4.1R possess some unique feature that is regulated distinctly by 4.1R. Disruption of this function in $4.1R^{-/-}$ mice produces neurobehavioral deficits that are consistent with the localization of 4.1R mRNA. Behavioral and neuroanatomical discriminations in rodents are sufficiently imprecise that the behavioral deficits we have observed cannot be ascribed with certainty to the brain structures enriched in 4.1R. Nonetheless, the highly selective enrichment of 4.1R in the dentate gyrus and the cerebellum implies that the observed behavioral defects in the $4.1R^{-/-}$ mice reflect the loss of 4.1R in these brain regions.

Materials and methods

In situ *hybridization*

In situ hybridization was performed as previously described [14] on 4-day-old whole-mount sections of wild-type mice and adult brain sections from $4.1R^{+/+}$ and $4.1R^{-/-}$ mice. The digoxigenin-labeled cRNAs correspond to mouse 4.1R [15] amino acids 481–842 (1083 bp; Figures 1a,2a–d) and amino acids 159–228 (exon 4, 210 bp; Figure 2e–h), mouse 4.1G [16] amino acids 860–988 (384 bp; Figure 1b), and mouse 4.1N [9] amino acids 428–523 (285 bp; Figure 1c).

Sensorimotor tasks

Methodology for sensorimotor evaluation was as described [11].

Learning tasks

The spontaneous alternation 12-arm radial maze and T-maze tests were conducted as described [17].

Supplementary material
A table showing the physical data and sensorimotor performance of the mice is published with this paper on the internet.

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Supplementary material

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Table S1

Physical data and sensorimotor performance of $4.1R^{+/+}$, $4.1R^{+/-}$ and $4.1R^{-/-}$ mice.

	$4.1R^{+/+}$	$4.1R^{+/-}$	$4.1R^{-/-}$
Body mass (g)	30.3 ± 0.9	30.4 ± 1.3	31.7 ± 2.3
Body length (cm)	8.7 ± 0.3	9.1 ± 0.5	8.9 ± 0.4
% Displaying contact placement	100	100	100
% Displaying visual placement	100	100	100
Latency to sniff cookie (sec)	223 ± 49	386 ± 61	285 ± 74
Open field			
Time spent in open field (sec)	60 ± 10	77 ± 39	100 ± 44
Number of lines crossed	211 ± 16	141 ± 31	151 ± 29
Elevated plus maze			
Time spent in open arms (min)	8.4 ± 4.5	2.6 ± 1.9	70 ± 3.0
Number of fecal boli	2.2 ± 0.5	2.5 ± 0.6	2.8 ± 0.5

All tests and measurements were conducted on 12 female mice of each genetic makeup and scored blindly. For sensorimotor evaluation, each mouse was subjected to three trials of each test. Error values are standard errors of the mean. There were no significant differences among the mice in body mass (g) or length (cm, snout to anus). All animals achieved the maximum score (100%) in contact and visual placement testing, which assess the ability to orient using tactile reflexes and visual cues, respectively. There were no significant differences among the animals in olfaction, as determined by the latency (sec) to locate a hidden cookie. Anxiety and activity levels were measured using open field testing [S1] and are reflected by time spent in the open field (sec) and the number of lines crossed, respectively. Anxious animals tend to spend less time in the open field and have decreased activity. The mice show no significant differences in these measures. Anxiety was also evaluated using an elevated plus maze [S1]. Anxious mice tend to spend more time (min) in the closed arms rather than the open arms of the maze and have an increased number of fecal boli. Again, there were no statistically significant differences among $4.1R^{+/+}$, $4.1R^{+/-}$ and $4.1R^{-/-}$ mice.

Reference

S1. Barnes CA, Nadel L, Honig WK: Spatial memory deficits in senescent rats. *Can J Psych* 1980, 34:29-39.