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Targeted Deletion of the Gene Encoding the La Autoantigen (Sjögren's Syndrome Antigen B) in B Cells or the Frontal Brain Causes Extensive Tissue Loss

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La antigen (Sjögren's syndrome antigen B) is a phosphoprotein associated with nascent precursor tRNAs and other RNAs, and it is targeted by autoantibodies in patients with Sjögren's syndrome, systemic lupus erythematosus, and neonatal lupus. Increased levels of La are associated with leukemias and other cancers, and various viruses usurp La to promote their replication. Yeast cells (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) genetically depleted of La grow and proliferate, whereas deletion from mice causes early embryonic lethality, raising the question of whether La is required by mammalian cells generally or only to surpass a developmental stage. We developed a conditional La allele and used it in mice that express Cre recombinase in either B cell progenitors or the forebrain. B cell *Mb1*^{Cre} La-deleted mice produce no B cells. Consistent with α*CamKII* Cre, which induces deletion in hippocampal CA1 cells in the third postnatal week and later throughout the neocortex, brains develop normally in La-deleted mice until ~5 weeks and then lose a large amount of forebrain cells and mass, with evidence of altered pre-tRNA processing. The data indicate that La is required not only in proliferating cells but also in nondividing postmitotic cells. Thus, La is essential in different cell types and required for normal development of various tissue types.

La is a conserved RNA binding protein that is found associated with a variety of small nascent RNAs that share a 3′-terminal oligo(U) tract that results from transcription termination by RNA polymerase III (Pol III) (reviewed in references 1 and 2). The most abundant of these are precursors to tRNAs, followed by pre-5S rRNA, pre-U6 snRNA, pre-7SK RNA, and others (reviewed in reference 3). Vertebrate La proteins are also found bound to and can modulate the translation of mRNAs that contain internal ribosome entry sites (IRES) or other complex 5′ regulatory motifs (*MDM2* mRNA), as well as mRNAs with 5′-terminal oligopyrimidine (5′-TOP) motifs that encode ribosome subunits and translation factors (4–10). La proteins are ubiquitous in eukaryotes and have been biochemically and/or genetically characterized in a wide variety of distant species (2, 11–13). While La has been implicated in many RNA-related pathways, its most established roles are in sequence-specific binding to the UUU-OH 3′ ends of pre-tRNAs and other small RNAs, resulting in their stabilization and protection from exonucleolytic digestion, and in chaperoning during their intranuclear processing and maturation (reviewed in references 1 and 14).

Although deletion of La from the distant yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* causes similar perturbations in pre-tRNA processing, as reflected by imbalances in pre-tRNA intermediates, La is nonessential under standard growth conditions (15–17). This imbalance due to deletion from *S. pombe* is associated with slow growth in minimal media and is accompanied by a stress response program of increased expression of amino acid and nitrogen metabolic genes (18). Although La may also interact with mRNAs in yeasts, this slow growth phenotype in *S. pombe* appears to be in response to alterations in nuclear pre-tRNA processing, because it can be suppressed by the overexpression of the tRNA nuclear export factor Los1 (Xportin-T, or XpoT, in mammals) (18). Aberrant nuclear pre-tRNA processing causes similar stress in *S. cerevisiae* that is suppressible by La (Lhp1) (19, 20). Especially noteworthy here is that recent work has indicated that RNA fragments derived from aberrant pre-tRNA processing induce stress-mediated loss of motor neurons in mice (21).

Association of human *MDM2* mRNA with La promotes *MDM2* translation with a consequent decrease in p53 protein and leukemia progression (22, 23). Other evidence suggests that La is a positive factor in the growth of some solid tumors (24–26). There is also a rich history of La association with viral RNAs as a positive factor in virus replication (5, 27–31). Small interfering RNA-mediated knockdown of La leads to a modest decrease in HeLa cell survival and more significant growth defects in protozoa (32, 33). *Drosophila melanogaster* with La deleted develops to a late larval stage (34). By contrast, mice carrying a conventional La knockout allele perish much earlier, at the blastocyst stage, and fail to produce embryonic stem cells (35). Thus, it would appear that different eukaryotes and perhaps different cell and tissue types rely on

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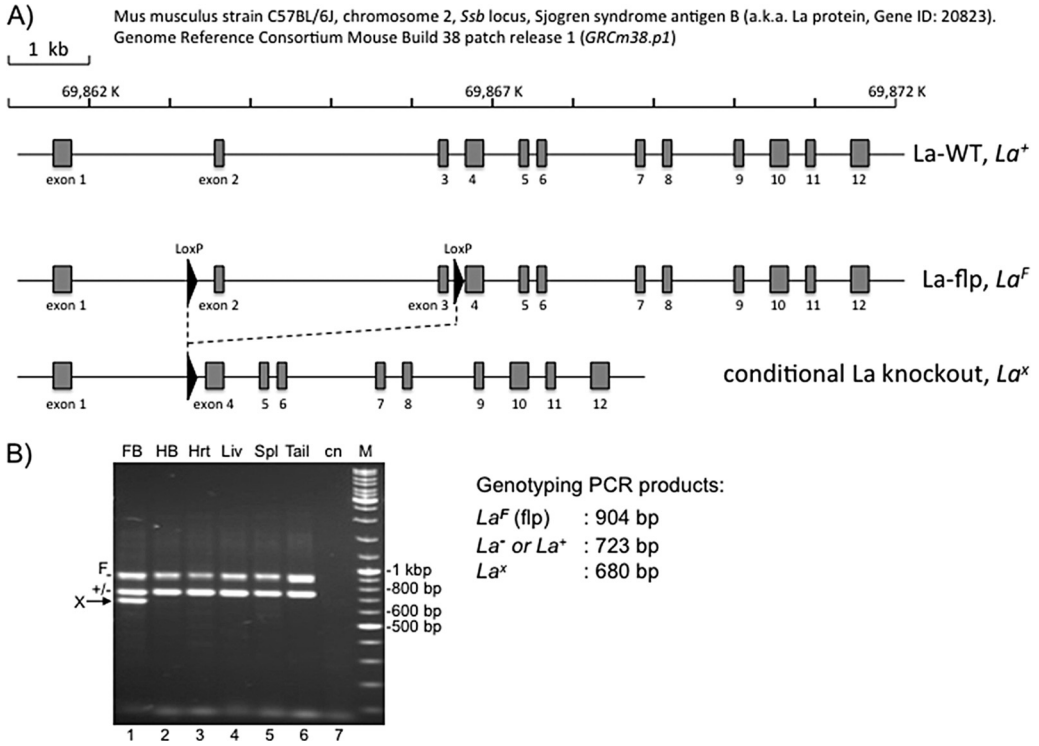

FIG 1 A conditional La allele. (A) Schematic representation of La (*Ssb*) alleles. (B) Tissue-specific detection of α*CaMKII* Cre-mediated rearrangement in a 12-week-old $La^{F/-}$ Cre^{CaMKII} mouse. FB, frontal brain; HB, hindbrain; Hrt, heart; Liv, liver; Spl, spleen; cn, control, in which no DNA was added to the PCR mixture; Lane M, molecular size markers. Lengths of the expected PCR products are indicated on the right.

La to promote proliferation and/or development to different extents.

Mammalian La (Sjögren's syndrome antigen B, encoded by *Ssb*) is expressed ubiquitously, consistent with a housekeeping function(s) (35, 36). Other investigators have used conditional alleles to examine requirements for housekeeping factors whose general deletion causes embryonic lethality (37). We chose to examine the effects of conditional La deletion in two distinct cell types, neurons of the forebrain and hematopoietic B cells. For this purpose, we created a floxed allele of La and used it with the Mb1 Cre recombinase for conditional deletion of La specifically in the B cell lineage. These mice had reduced spleen sizes with no detectable B cells, no serum IgG, and a complete block in B cell development at an early pro-B stage. Another approach was to use Cre expressed from the α*CamKII* promoter, which is induced soon after birth and restricted to the forebrain. This study revealed that brain size was normal until 5 weeks postpartum, followed by progressive loss of forebrain tissue. The La-deficient brains lose neurons with age, arguing that La is required for cell survival in postmitotic neurons. We also found that imbalances in pre-tRNA intermediates, similar to those observed in La-deleted yeast cells, also occur in the forebrains of La-deleted mice.

MATERIALS AND METHODS

Mice. All mouse studies were performed at the NIH under protocol ASP 10-005, approved by the IACUC of NICHD, and at the Icahn School of Medicine at Mount Sinai under IACUC protocol 02-0220. The La conditional allele was created by Ingenious Targeting Laboratory, Inc., Stony Brook, NY. The original allele contained a Neo^r cassette flanked by flippase recombination target (*FRT*) sites in intron 3. Transcription from the Neo^r promoter was antisense relative to La. This allele conferred no apparent phenotype in heterozygous form but was embryonic lethal when homozygous. Removal of Neo^r by crossing with mice carrying the Flp recombinase produced the La allele, La^F, containing *loxP* sites (Fig. 1). La^F confers no apparent phenotype in the heterozygous ($La^{+/F}$) or homozygous ($La^{F/F}$) form or as a compound heterozygote with the conventional knockout allele ($La^{F/-}$). All brains and/or sections designated $La^{F/-}$ Cre^{CaMKII} were demonstrated to be $La^{x/-}$ Cre^{CaMKII} by PCR genotyping. The $Mb1^{Cre/+}$ mice were obtained from Michael Reth (Max Planck Institute of Immunology and Epigenetics, Freiburg, Germany). The α*CaMKII* Cre mice were obtained from the Jackson Laboratory [B6.Cg-Tg(Camk2a-cre)T29-1Stl/J].

Immunohistochemistry of hippocampus sections. Mice were deeply anesthetized with isoflurane and decapitated, followed by rapid removal of the brain, which was placed in 10% neutral phosphate-buffered formalin for 24 to 48 h. Following fixation, brains were embedded in paraffin and sliced into 4-μm sections, which were deparaffinized by heating at 60°C for 30 min, followed by two 5-min immersions in xylene. The sections were rehydrated through a graded series of alcohols, followed by two 15-min rinses in distilled water. For antigen retrieval, the slides were heated in a pressure cooker for 4 min in 50 mM citrate buffer (pH 5.6). Thereafter, they were washed twice for 15 min each in phosphate-buffered saline (PBS; Sigma). Antibodies against mLa (1:2,000) and tubulin (1:500; Sigma) were prepared in PBS containing bovine serum albumin and NaN_3 (Invitrogen), and the sections were incubated at room temperature for 2 h. As negative controls, the primary antibodies were omitted. The Ultra Vision LP detection system (ThermoScientific) was used for diaminobenzidine (DAB) staining according to the manufacturer's protocol.

Primers used for genotyping. Primers LAXmid2 and LAXA62 were used to generate PCR products of 723 bp for the La^+ allele and 904 bp for the La^F allele; LAXlox67 and LAXA62 were used to generate a 680-bp

product after Cre-mediated rearrangement, representing the La^x allele; oIMR1084 and oIMR1085 generated 101-bp products in the presence of Cre^+. Primer sequences were as follows: LAXmid2, GAGGATTGAGCC CAACTCCTCAGTTT; LAXA62, GGGAGTGGTCTGCTTGGTGATC; LAXlox67, GGCACATCCTTACCTTCCAGCGTCCC; LAXA62, GGGA GTGGTCTGCTTGGTGATC; oIMR1085cre, GTGAAACAGCATTGCT GTCACTT; oIMR1084cre, GCGGTCTGGCAGTAAAAACTATC.

For some procedures, mice were subjected to perfusion-fixation before dissection. It should be noted that this procedure has a dehydrating effect and leads to brain weights that are significantly lower (~10%) than those after direct isolation of "wet" brains without prior perfusion-fixation (data not shown). The blood of anesthetized mice was replaced with PBS, 4% paraformaldehyde. Brain sectioning as well as hematoxylin and eosin (H&E) and Nissl staining were performed using standard procedures (Histoserv Labs, Germantown, MD). Anti-mLa antibody raised in rabbits against full-length purified recombinant mLa with a C-terminal His_6 tag was previously described (35). Stained brain sections were converted into digital whole-slide images by using an Aperio GL scanner and analyzed using ImageScope software (Aperio) (38).

IgG purification. Two hundred microliters of blood collected in EDTA was centrifuged for 5 min at 13,000 rpm. Ten microliters of the supernatant was added to 200 μl of DPBS (Dulbecco's phosphate-buffered saline) and incubated with 15 μl protein A-Sepharose. The supernatant was removed, resin was washed with DPBS, and bound material was eluted with 20 μl 100 mM glycine (pH 3.0), followed by neutralization with 3 μl 1 M Tris-HCl (pH 8.8). Five microliters was examined on an SDS gel stained with Coomassie blue.

Flow cytometry. Single-cell suspensions were prepared from bone marrow (BM), spleen, and peritoneal cavity (PerC) washouts of 2- to 3-month-old mice by using established procedures (39). The cells were incubated in Hanks balanced salt solution (HBSS) supplemented with 3% fetal bovine serum (FBS) with 2.4G2 antibody (Ab) to block Fc receptors, followed by staining with antibodies against B220 (RA3-6B2), IgM (II/41), CD43 (S7), CD4 (GK1.5), CD8 (53-6.7), CD19 (ID3), and CD5 (53-7.3). These Abs were purchased from BD Biosciences and Biolegend and were directly conjugated with fluorochromes. After washing, the cells were resuspended in HBSS–3% FBS with Sytox AADvanced dead cell stain (Invitrogen) and were analyzed by using an LSR II analyzer (BD Biosciences). The data were analyzed using FlowJo software (Tree Star, Inc.).

RNA analysis. Brains were isolated immediately following euthanization. The frontal cortex was dissected from the rest of the brain and immediately homogenized using a Dounce homogenizer containing ice-cold TRIzol (~1 ml/50 mg of brain tissue). Total RNA was extracted after addition of 200 μl chloroform/ml TRIzol and precipitated with ethanol and sodium acetate according to standard procedures. The total RNAs were electrophoresed on polyacrylamide gels containing 8 M urea, transferred to a nylon membrane (Gene Screen Plus), and probed with ^{32}P-5′-end-labeled oligonucleotide DNAs (Lofstrand Labs, Gaithersburg, MD) according to standard procedures. Quantification was performed by using a Fuji PhosphorImager.

RESULTS

The conditional *La* allele. Exon 2 contains the natural AUG start site of translation and was deleted in our conventional knockout allele, La^- (35) (Table 1). The wild-type, floxed, and conditional La alleles, La^+, La^F, and La^x, respectively, are shown in Fig. 1A. After the action of Cre recombinase, the La^F allele's exons 2 and 3 are deleted, which encode amino acids 1 to 57 of the La protein, comprising most of the La motif and all of its sequence-specific RNA binding elements (40, 41), resulting in the La^x allele (Table 1).

$La^{+/-}$ heterozygous mice with a wild-type allele (La^+) and a conventional knockout allele (La^-) exhibit no obvious phenotype (35). As expected, $La^{F/-}$ mice are similarly unaffected. We took advantage of the use of $La^{F/-}$ mice with Cre, so that only one allele rather than two would have to be acted on by Cre to produce nullizygous La ($La^{x/-}$) cells.

TABLE 1 Alleles evaluated in this study

Allele description	Designation
La knockout	La^-
La wild type	La^+
La flip (before Cre)	La^F
La floxed (after Cre)	La^x
Mb1 Cre	$Mb1^{Cre}$
Mb1 wild type	$Mb1^+$
α*CaMKII* Cre	Cre^{CaMKII}

We first describe the effects of forebrain-specific α*CamKII* Cre. *In situ* hybridization data from Allen Brain Atlas, showing the spatial expression of La (Ssb) mRNA in the brain of a 56-day-old C57BL/6J mouse, are available online (http://mouse.brain-map.org/experiment/show/70429459). The highest La (Ssb) mRNA expression levels are found in the hippocampal formation, including cells of the CA1 region and dentate gyrus (DG), isocortex, cortical subplate, and olfactory areas. Other brain regions, such as the pons, medulla, white matter of the cerebellum, and regions immediately adjacent to the CA1 and DG cells, have relatively low expression levels of La (Ssb) mRNA.

La deletion by α*CamKII* Cre causes reduction in the La protein in targeted brain cells. We crossed our mice with B6.Cg-Tg(Camk2a-cre)T29-1 mice (42) to produce $La^{x/-}$ Cre^{CaMKII} mice and control sibling offspring. Consistent with the temporal activity of the α*CamKII* promoter, recombination of floxed alleles occurs in Camk2a-cre-T29-1 mice during the third postnatal week in the forebrain, in CA1 pyramidal cells of the hippocampus, neocortex, dentate gyrus, and striatum, but not in the hindbrain (brain stem and cerebellum) (42, 43). Figure 1B shows our PCR results that confirmed the tissue specificity of the α*CamKII* Cre-rearranged *La* allele (band X, La^x) in the forebrain only.

The CA1 cell region of the hippocampus of mouse brains at 11 weeks of age was examined by using immunohistochemistry (Fig. 2A). A moderate range of signal intensity was observed in the CA1 region after staining with anti-mLa antibody in control mice, but significantly less staining was seen in the Cre-deleted La mice (Fig. 2A, top row, mLa panels). While most cells in La-deleted mice showed less mLa than those of control siblings, a small fraction of the cells showed similar signal intensities in *La*-deleted mice as in the control siblings. There were no gross morphological differences among the *La*-deleted and control mice after staining with H&E or antitubulin antibodies (Fig. 2A, middle and bottom rows). The results indicated that the La^F allele was subjected to Cre-mediated rearrangement with loss of La expression in the Cre^{CaMKII}-targeted hippocampal CA1 cells.

***La* deletion by α*CamKII* Cre causes postnatal loss of forebrain mass.** We monitored several cohorts of $La^{F/-}$ Cre^{CaMKII} mice and their control sibling offspring over time. We observed no consistent obvious phenotypic differences in gait or other behaviors in the *La*-deleted mice as they aged. However, inspection of the brains of 59-week-old La-deleted and control mice revealed a striking deficiency of forebrain cortex, with no significant differences in the hindbrains (Fig. 2B). Effects on the cerebral cortex would be unexpected based on the expression pattern of Cre^{CaMKII} in young mice (42). However, this Cre^{CaMKII} allele (i.e., in B6.Cg-

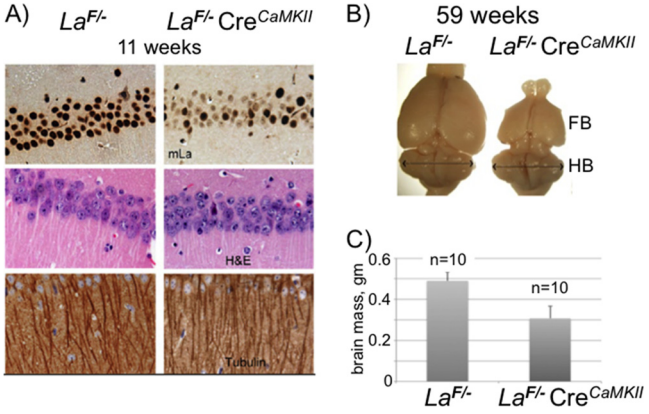

FIG 2 Reduction in La protein levels and decreased frontal brain mass following targeted deletion of La. (A) Immunohistochemistry of the hippocampal CA1 regions of 11-week-old α*CamKII*^{Cre} *La*-deleted and control mice, as indicated. (Upper row) Immunohistochemistry with anti-mLa antibody (35); (middle row) H&E stained section; (bottom row) antitubulin antibody-stained section. (B) α*CamKII*^{Cre} *La*-deleted and control mice brains as indicated. Double-sided arrows superimposed on the hindbrains (HB) are the same size; the FB (front brain) is also indicated. (C) Comparison of average brain wet weights of 10 α*CamKII*^{Cre} *La*-deleted and 10 control mice. Error bars indicate standard deviations. $P = 1.2 \times 10^{-5}$. All of these samples were obtained without prior perfusion-fixation.

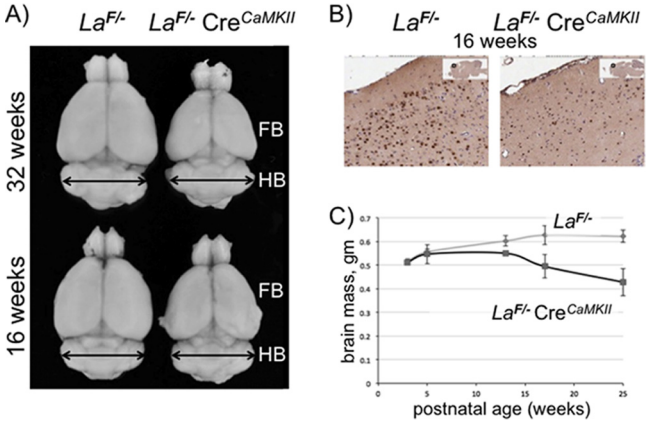

FIG 3 *La* deletion in α*CamKII*^{Cre} animals causes developmental postnatal loss of frontal brain mass. (A) Development of α*CamKII*^{Cre} *La*-deleted and sibling control brains at 16 and 32 postnatal weeks. Double-sided arrows on the hindbrain (HB) images are the same size; FB, front brain. (B) Immunohistochemistry of neocortex stained with anti-mLa antibody and H&E counterstaining of 16-week-old mouse cortices. (C) Plot of brain mass versus age of α*CamKII*^{Cre} *La*-deleted and control mice. For mice aged 13 weeks and older, *P* values ranged from 0.004 (13 weeks) to 0.0001 (17 and 25 weeks). All of the samples were obtained by dissection after perfusion-fixation.

Tg(Camk2a-cre)T29-1 mice) is highly active for Cre-mediated recombination throughout the cortex in older mice that also carry the Rosa26 β-galactosidase reporter (44) and a human alkaline phosphatase reporter (45), which would nicely account for our observations of decreased cortex specifically (Fig. 2B). We compared brain masses in 59- to 79-week-old $La^{F/-}$ Cre^{CaMKII} mice and their age-matched siblings; the *La*-deleted brains weighed significantly less than those of controls (Fig. 2C). We did not subject mice to systematic physiological or behavioral testing (see Discussion).

Inspections of brains from 16- and 32-week-old mice revealed evidence of progressive differences in forebrain size (Fig. 3A). Immunohistochemistry of the cortex at 16 weeks showed decreased *La* expression and cellularity in $La^{F/-}$ Cre^{CaMKII} mice. In addition to less-intense mLa staining (Fig. 3B), H&E staining revealed a decrease in neuronal density and loss of cortical cytoarchitecture in the La-deficient brain (data not shown, but see below).

A comparison of total brain mass obtained after perfusion-fixation for La^{F-} Cre^{CaMKII} mice and sibling controls from 3 to 25 weeks of age is shown in Fig. 3C. The brain weights of our control mice ($La^{F/-}$) basically agreed with results from previous studies (46). The brain mass plot revealed that consistent with the temporal activity of Cre^{CaMKII}, a difference in brain mass first appeared to distinguish *La*-deleted and control mice after 5 weeks. Remarkably, for several weeks thereafter, a time when the normal developing mouse brain transitions from a state of growth to a more constant steady-state level (46) (Fig. 3C, gray line), the $La^{F/-}$ Cre^{CaMKII} mouse brains began to lose mass (Fig. 3B, black line). The rapid decline observed from 13 to 25 weeks in $La^{F/-}$ Cre^{CaMKII} mice slowed significantly thereafter (Fig. 2C).

Progressive cell loss in the forebrains of $La^{F/-}$ Cre^{CaMKII} mice. Histopathological analysis using H&E as well as Nissl staining revealed age-dependent cortical atrophy and loss of hippocampal CA1 cells in the La-deficient mouse brains (Fig. 4A to F). While the cortex was of similar thickness in 12-week-old mice (Fig. 4A and B, left panels), La-deficient mice began to exhibit gross differences in appearance of the frontal cortex and its size by 16 weeks (Fig. 4C and D, left panels). In 65-week-old mice, the cortex of La-deficient brains was severely diminished compared to controls (Fig. 4E and F, left panels), consistent with the decreased forebrain seen in Fig. 2B. In the hippocampus, the pyramidal cell layer of area CA1 and subiculum progressively thinned over time, while area CA3 and the dentate gyrus were relatively spared (see below).

We also examined La protein staining and cellularity in the same brain preparations, using anti-mLa Ab and counterstaining with H&E. Figure 4G and H show high magnifications of the control and La mutant brains, respectively, in the somatosensory cortex. The control brain clearly showed more La content (Fig. 4G) than the La mutant (Fig. 4H).

Figure 4I and J show control and La mutant brains, respectively, in the retrosplenial cortex. Again, the control brain had more La content (Fig. 4I) than the mutant (Fig. 4J). Interestingly, unlike the somatosensory cortex, while the control retrosplenial cortex showed normal cell distribution and the La mutant brain contained less La, there were a large number of cells that appeared to express low levels of La protein (see Discussion).

The apparent sparing of the dentate gyrus in the La mutants, while the CA1 cells were diminished, as observed by H&E staining (Fig. 4A to F, DG), was interesting because the DG was targeted by Cre^{CaMKII} along with the CA1 (44). Figure 4K and L show high magnifications of both the CA1 and DG regions, side by side in control and La mutant mice. In control mice, most of the CA1 cells contained La (brown cells), and there were few if any that were devoid of La (Fig. 4K, middle panel, CA1, enlarged circle). In contrast, numerous cells in the DG contained less La (blue cells) in control mice (Fig. 4K, lower panel, DG, enlarged circle). Figure 4L shows the same regions in the La mutant mice.

The cumulative data indicate that as the number of La-con-

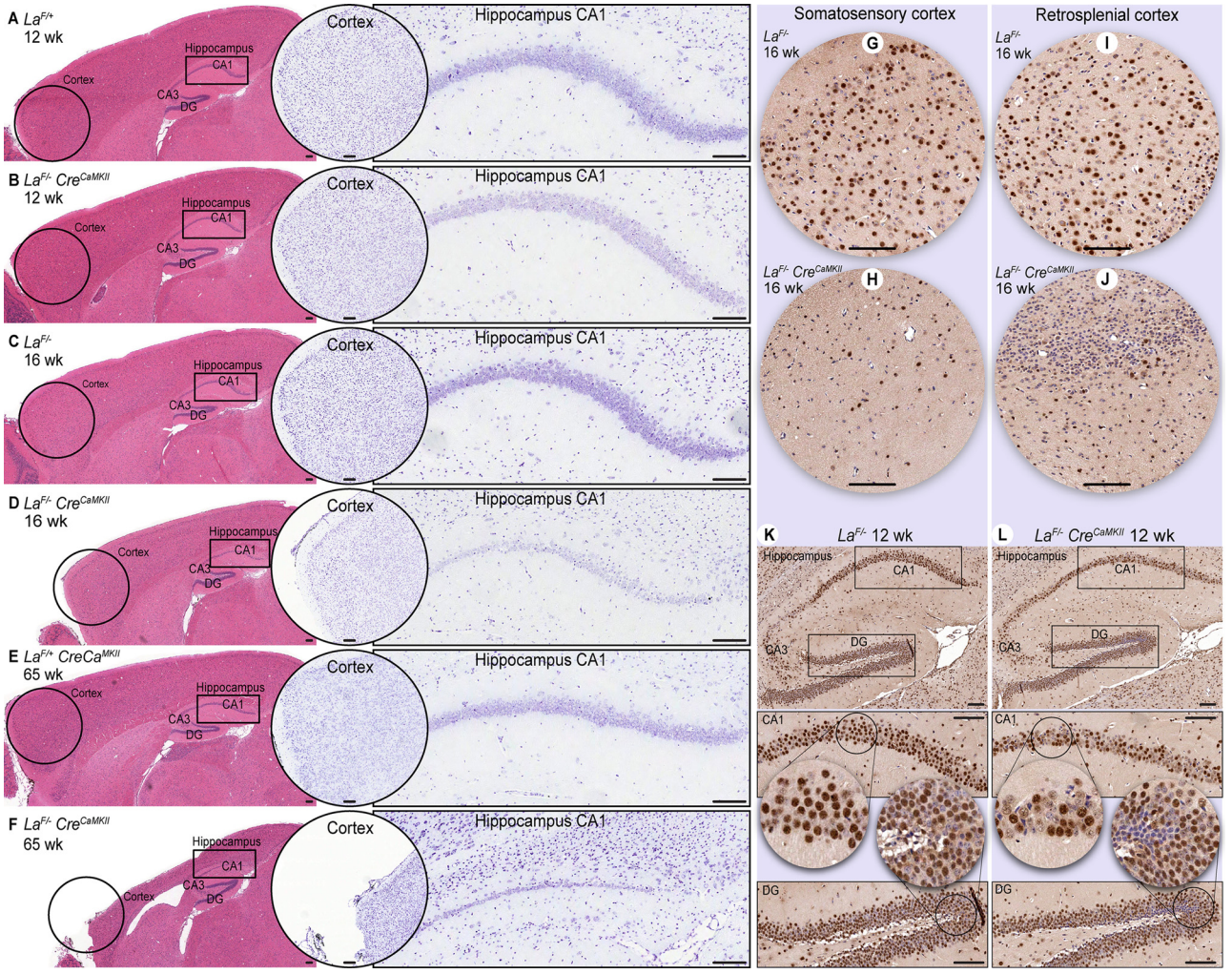

FIG 4 Progressive neurodegeneration in the cerebral cortex and hippocampus of *La*-deleted mice. Representative sections of the forebrains from the *La*-deficient ($La^{F/-}$ Cre^{CaMKII}) and control littermates ($La^{F/-}$) at 12 weeks (A and B), 16 weeks (C and D), and 65 weeks (E and F). Areas of the frontal cortex and hippocampus (CA1 cells) that are outlined in the overview images (H&E, left panels) are shown at a higher magnification in the adjacent, corresponding Nissl-stained sections. Note there was some decrease in cortical thickness and a more pronounced decrease in neuronal density in the hippocampus (CA1) of 16-week-old *La*-deleted mice than in control littermates. Cortical atrophy and neuron loss in the hippocampus (CA1) were especially pronounced in 65-week-old *La*-deleted mice compared to controls (compare panels F and E). Bars in the Nissl insets (cortex and hippocampus), 100 μm. (G to J) High-magnification immunohistochemistry images of representative regions of the somatosensory cortex (G and H) and retrosplenial cortex (I and J), stained with anti-mLa antibody and H&E counterstaining, from 16-week-old control brains (upper panels) and matched littermate *La* knockout mutant brains (lower panels). (K and L) High-magnification immunohistochemistry results for the hippocampal regions of 12-week-old control (K) and matched littermate *La* knockout mutant (L) mouse brains stained with anti-mLa antibody and H&E counterstaining. The upper panels show low magnification images, which were further magnified for the middle and lower panels, including the circular insets, as annotated.

taining cells decreases with age due to Cre activity in the cerebral cortex and hippocampal CA1 cells of *La*-deleted mice, so do the total number of cells and the overall mass in these regions. In the DG, one of the few regions of neurogenesis in adults, there is much less net loss of cells (see Discussion).

La deletion by Mb1 Cre causes hyposplenia, severe B cell lymphopenia, and agammaglobulenemia. To determine if expression of La is also critical to hematopoietic differentiation, we crossed $La^{F/-}$ mice with mice expressing Mb1 *Cre* (37), which is strongly expressed in the B cell lineage beginning at the early pro-B cell stage of development in the bone marrow (BM) and persists up to the stage of plasma cell differentiation in peripheral lymphoid tissues, including the spleen (47). In normal spleens, about 50% of all lymphocytes are B cells. At necropsy, the spleens of *La*-deleted mice were visually smaller than those of control mice and averaged about half the weight of spleens from controls (Fig. 5A). We also tested for the presence of IgG by SDS-PAGE separation of serum proteins eluted from a protein A-Sepharose column that selectively bound IgG and, nonspecifically, small amounts of albumin. Bands consistent with the sizes of IgG heavy chain (IgH) and light chain (IgL) (Fig. 5B) were readily detected in samples generated from sera of control (lane 1) but not *La*-deleted (lane 2) mice. (Additional studies of the two cohorts revealed an absence of IgM in serum of *La*-deleted mice, as determined by enzyme-linked immunosorbent assay). This indicated that $La^{F/-}$ $Mb1^{Cre/+}$ mice are unable to generate mature immunoglobulin-secreting cells.

To determine the stage at which B cell differentiation was

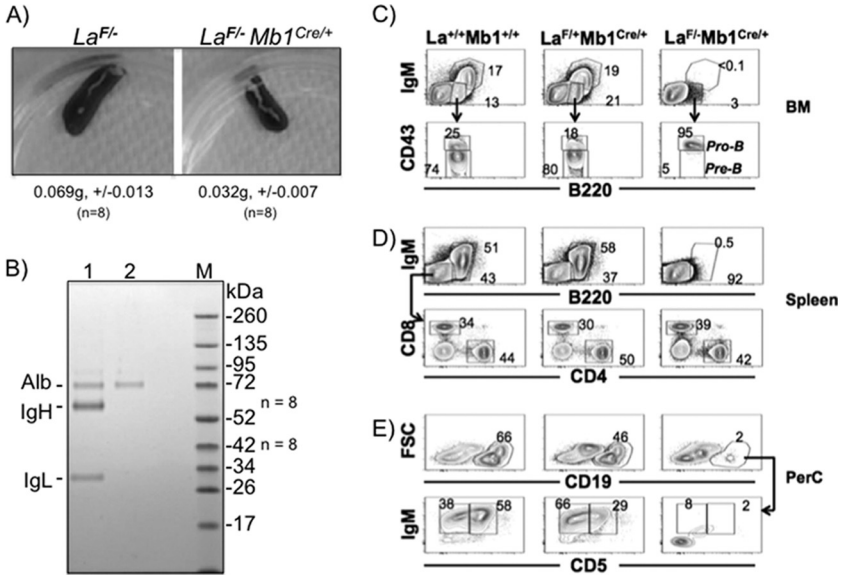

FIG 5 *La* deletion by *Mb1*^{Cre} causes reduced spleen size, agammaglobulenemia, and absence of cells of the B lineage. (A) Photographs of freshly isolated spleens from typical control and *La*-deleted 17-week-old mice. The mean ± standard deviation spleen weights from eight control and eight *La*-deleted mice are indicated under the images. (B) Absence of serum IgG in *La*-deleted mice. Lane 1, control mouse serum; lane 2, from *La*-deleted mouse serum. (C to E) Developmental arrest of La-depleted B lineage cells. BM (C), spleen (D), and PerC cells (E) were stained with the indicated Abs and analyzed by flow cytometry using the indicated gating parameters. The numbers are frequencies of cells falling in each gate. Data are representative of multiple mice with similar results. (C) BM IgM$^+$ B220^{lo} cells (top panels) were gated to identify pro-B cells (CD43$^+$ B220$^+$) and pre-B cells (CD43$^-$ B220$^+$) (lower panels). (D) Spleen cells were stained to identify B cells (IgM$^+$ B220$^+$) and non-B cells (IgM$^-$ B220$^-$) (top panels). (E) PerC cells were examined using the gating parameters shown.

blocked in La-deficient mice, we used flow cytometry to study the composition of different compartments of the B cell lineage in the BM and spleen and among PerC cells by using the gating parameters shown in Fig. 5C to E. Studies of BM cells revealed an essentially total block in B cell development at the pro-B cell (B220$^+$ CD43$^+$)–to–pre-B cell (B220$^+$ CD43$^-$) transition (Fig. 5C), precisely the time at which Mb1 is first expressed in B cell development (47). Predictably, this block resulted in the complete absence of IgM-expressing B cells in both the spleen (Fig. 5D) and PerC (Fig. 5E). In contrast, the frequencies of splenic CD4$^+$ and CD8$^+$ T cells were completely normal in La-deficient mice, consistent with a lack of Mb1 expression in the T cell lineage.

Evidence of altered pre-tRNA processing in the mutated *La* brain. It is well established that La protein in human and mouse cells is bound to pre-tRNAs and other nascent Pol III transcripts (48–50). However, the studies showing that La contributes functionally to pre-tRNA maturation utilized distant yeasts, *S. cerevisiae* and *S. pombe*, and indicated its involvement at multiple points in the pathway. Maturation of a nascent precursor tRNA occurs along a complex pathway, requiring different end-processing activities to remove the 5′ leader and 3′ trailer, as well as splicing for tRNAs with introns (and many base and nucleotide modifications), the precise order of which may vary for different pre-tRNAs (reviewed in references 14, 15, 51, and 52). Moreover, these activities are directed so as to prevent end-retaining pre-tRNAs from entering the cytoplasm. La protein imposes order on the pre-tRNA processing pathway (reviewed in references 14, 15, 17, and 53). For some pre-tRNAs, La increases the processing efficiency with which the nascent transcript is converted to the next intermediate in the pathway (16, 17). In addition to stabilizing nascent pre-tRNAs bearing a 3′ oligo(U) tract, La also retains these in the nucleus, affording them access to efficient and ordered end processing (53, 54). Moreover, pre-tRNAs with structural impairments require a distinct RNA chaperone-like activity of La for efficient maturation (55–57).

However, there is no evidence of which we are aware that La is functionally involved in pre-tRNA metabolism in higher eukaryotes. Therefore, we examined the pre-tRNA intermediates in the forebrains of control and La mutant mice. Analysis of intron-containing pre-tRNAs is a standard way to characterize the effects of La protein on pre-tRNA metabolism (15–17, 53, 54, 56, 57). Figure 6A and B show Northern blot results for RNA isolated from the forebrains of control and Cre^{CaMKII} La mutant mice, performed with ^{32}P-labeled oligonucleotide-DNA probes complementary to either the 3′ trailer [including the 3′ oligo(U)] or the intron of pre-tRNA^{Tyr}, respectively. The 3′ trailer probe (Fig. 6A) showed two RNA species in control and La mutant forebrains: an upper band reflecting nascent pre-tRNA with a 3′ trailer and a lower band. The lower band represented a processing intermediate that retained its 3′ trailer but appeared to have been spliced, because after stripping (data not shown), the intron probe on the same blot detected only the upper band of the two bands (Fig. 6B). We concluded from these data that the lower band in Fig. 6A represents a pre-tRNA^{Tyr} processing intermediate that retains its 3′ trailer-containing intermediate and whose intron was removed. Most significant was the obvious paucity of the lower, 3′ trailer-containing intermediate in the La mutant forebrain relative to the control forebrain (Fig. 6A). The blot was next subjected to a probe complementary to U12 snRNA, a Pol II transcript, to serve as a loading control (Fig. 6C). Similar differences in the ratios of lower to upper bands in control and La mutant forebrains were observed in other sample sets; these were quantified and are plotted in Fig. 6D. These data provide clear evidence that the La-deficient forebrains exhibited significant reductions in the accumulation of the

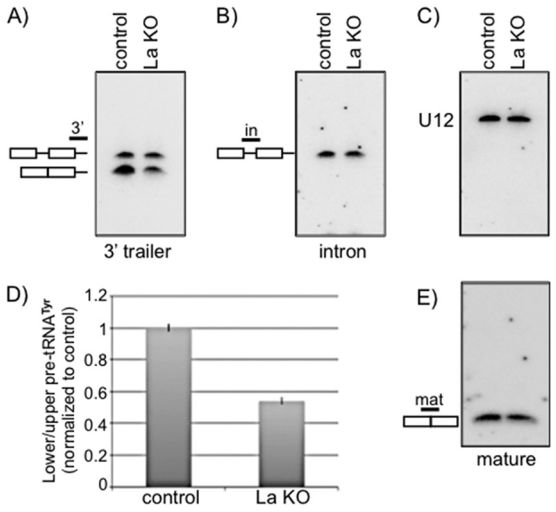

FIG 6 Northern blot analysis revealed altered pre-tRNA metabolism in *La* mutant forebrains. (A to C) A blot containing total RNA isolated from the frontal cortex was probed, autoradiographed, stripped, and reprobed with probes to detect different intermediates in the maturation pathway for pre-tRNA^{Tyr}, as indicated. The cartoons to the left of panels A and B are schematic representations of the species detected; rectangles reflect exons, lines reflect the intron or 3′ trailer, and the thick line reflects the position of the probe. (C) The same blot, probed for U12 snRNA to serve as a loading control. (D) Graph of the ratio of lower versus upper band intensities from panel A and a similar probing of another matched sample set. Error bars reflect results of two independent experiments from different sample sets. (E) The same blot, probed for spliced mature tRNA^{Tyr}.

3′ trailer-containing pre-tRNA^{Tyr} intermediate relative to control forebrains. These data reflect alterations of pre-tRNA^{Tyr} metabolism caused by La knockout in developing mouse brains.

We probed the same blot for the mature tRNA^{Tyr} (Fig. 6E). The amount of mature tRNA^{Tyr} appeared lower in the La knockout samples than in the control sample (Fig. 6C, U12 RNA). However, quantification of these and other matched samples revealed only a small decrease in the amount of mature tRNA^{Tyr} relative to U12 in the La knockout forebrains (data not shown), reminiscent of previous findings with *La*-deleted yeast (see Discussion).

DISCUSSION

It was unknown whether a cell-type-specific conditional genetic deletion of *La* would be detrimental to mouse B cells or brain cells. We found that conditional deletion of the *Ssb* gene encoding the La protein in B cell progenitors mediated by *Mb1*^{Cre} led to the complete absence of B cells and circulating immunoglobulins. The *Mb1*^{Cre} mice also had significant reductions in spleen size, as expected with total loss of B cells and consistent with the block in B cell development at the pro-B–to–pre-B cell transition in the bone marrow (Fig. 5, BM). This indicated that La is required for the development and/or viability of B cells. These studies revealed a critical, nonredundant requirement for the La protein beyond the stage at which expression was extinguished, the pro-B cell–to–pre-B cell transition.

In separate studies using Cre^{CaMKII} mice, we observed neuronal loss in the cerebral cortex and hippocampal CA1 region in conditional *La*-deleted adult mice, sites known to be nonneurogenic in the adult mouse brain (58). The cumulative results suggested that La is essential not only for proliferating cells but also for postmitotic cells.

Conditional deletion of *La* mediated by Cre^{CaMKII} led to loss of hippocampal CA1 cells and widespread loss from the forebrain, with continuing cortical atrophy thereafter. Although one would expect mice with decreased forebrain mass to be compromised in gait or other functions, we have not undertaken systematic testing, because to do so and achieve statistically significant results would require large numbers of matched littermates and detailed analyses to distinguish effects on motor function versus cognitive behavior.

That only fractions of the hippocampal CA1 and neocortex cells were depleted of La protein suggests, among other possibilities, that *CaMKII*-driven Cre was inactive in some of the cells. Consistent with this, when we used high-resolution imaging to examine β-galactosidase that resulted from Cre-mediated recombination in Rosa26 reporters carrying the B6.Cg-Tg(Camk2a-cre)T29-1 allele (44), we found that some cells in the cerebral cortex and CA1 region had escaped recombination (data not shown).

Not all of the brain regions targeted by Cre^{CaMKII} showed a net loss of cellularity. Intriguingly, this was most obvious in the DG, one of the few regions currently known to have high rates of neurogenesis in adults. Immunohistochemistry revealed a set of cells in the DG that contained low levels of La relative to adjacent cells (Fig. 4K and L, blue cells). Our analysis of the β-galactosidase reporter data from the *Cre*^{CaMKII} Rosa mice revealed that a significant amount of cells in the same region of the DG had not (yet) induced the *CaMKII* promoter that drives Cre expression (data not shown). Although the data might suggest an intricate relationship between induction of CaMKII, accumulation of La, and neurogenesis in the DG, examination of this relationship was beyond the scope of the present study.

La is nonessential in yeast but is essential in mice and fruit flies, albeit at different stages of development. Multiple points should be noted when considering this variability, in particular the essential requirements of yeast and metazoa. Although La is nonessential in yeast, its absence causes alterations of tRNA processing, and it becomes essential in the presence of structure-altering mutations in some tRNA genes that are otherwise nonlethal in its presence, consistent with an RNA chaperone-like function (55). Mammalian La proteins are larger and more complex than those of yeast and include an additional RNA recognition motif (RRM) (1). As noted in the introduction, mammalian La has been implicated in the regulation of multiple mRNAs, including those for housekeeping functions, such as ribosomal proteins. In addition, La has been implicated in the biogenesis of rRNA and microRNAs (miRNAs) (59–61). Thus, although we have demonstrated altered pre-tRNA metabolism in the *La* knockout brain, present technology does not allow us to determine if deficiency in this pathway alone is sufficient to cause cell lethality and the other phenotypes observed. The present data do not rule out the possibility that the essential function of mouse La involves a role in the metabolism of other classes of RNAs with which it has been found to be associated, the 5′-TOP mRNAs that encode ribosome subunits and related factors (4), MDM2 or other regulatory functions, rRNA biogenesis, or miRNA biogenesis.

Human La can replace the *in vivo* function of *S. pombe* La in three activities of pre-tRNA maturation: 3′ end protection, nuclear retention to afford efficient processing, and pre-tRNA chaperone activity for those pre-tRNAs that require assistance in folding (17, 53, 54, 56, 57). The RNA chaperone activity of La, which has been demonstrated for pre-tRNAs but may also work on

mRNAs, uses a mode of RNA recognition distinguishable from UUU-3′-OH binding (54, 62). In any case, the presence of an additional RRM suggests that mammalian La may have adopted a function beyond that of the yeast proteins. Such a function may involve an activity in addition to the binding and protection of pre-tRNAs and/or other RNA polymerase III transcripts that localizes to RRM2. RNA chaperone and other activities that appear to rely on RNA binding distinguishable from UUU-3′-OH binding may be involved (31, 62), affecting mRNA translation, as noted above, or an miRNA-related function (60, 61).

Alternatively, an additional RRM may allow more control or regulation over the pre-RNA or other polymerase III transcript binding activity, perhaps related to the pre-tRNA chaperone activity of RRM1 (17, 63). While *S. pombe* and *S. cerevisiae* contain 180 and 275 tRNA genes, respectively, mice and humans contain significantly more, 432 and 506. Thus, it seems reasonable to expect that some pre-tRNAs in mice and humans may require the pre-tRNA-related activities of La to a greater extent than yeast. As La activities in the pre-tRNA, mRNA, and miRNA pathways have been reported to rely to various degrees on the second RRM, it will be of interest in future work to examine the extent to which different La constructs rescue the phenotypes observed here.

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