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Expression of the transcription factor regulatory factor X1 in the mouse brain

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Abstract: Limited information indicates that the regulatory factor X1 (RFX1), the prototype member of the transcription factor RFX family, may play a role in the central nervous system. Our recent study showed that knockout of the *Rfx1* gene led to early embryonic death. In the present study, we showed that heterozygous *Rfx1*$^{+/-}$ mice were fertile and grew normally. An abundant amount of RFX1 proteins were expressed in the olfactory bulb, hippocampus and cerebral cortex as detected by β-galactosidase staining (the gene knockout vector contained a coding region for β-galactosidase) and immunofluorescent staining with an anti-RFX1 antibody. RFX1 positive immunostaining was mainly in the nuclei of neurons and microglial cells and was absent from the astrocytes of mouse brains. The heterozygous *Rfx1*$^{+/-}$ mice expressed RFX1 mRNA and proteins at a level similar to that in the wild-type mice in the olfactory bulb and hippocampus. The expression level of RFX1 proteins was similar in the brains of mice ranging from 15 day old embryos to four month old adults. Our results suggest a significant expression of RFX1 proteins in the mammalian brain. This expression is cell-type and brain-region specific and may take a random monoallelic expression pattern. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 2, pp. 344–351*)

Key words: brain, microglial cells, mouse, neurons, regulatory factor X1

Introduction

The regulatory factor X (RFX) proteins are unique transcription factors that contain a highly conserved 76-amino acid DNA binding domain. This domain can bind X-box consensus sequences in the promoter regions of various genes [1]. Seven RFX proteins (named RXF1-7) have been identified to date in mammals [2]. Their functions are beginning to be understood. For example, RFX5 can regulate the expression of the major histocompatibility complex class II genes [3] and knockout of *Rfx3* gene causes severe ciliopathies leading to diabetes and left-right asymmetry specification [4, 5].

RFX1 is the prototype member of the RFX family. Our recent study showed that knockout of the *Rfx1* gene in mice leads to early embryonic lethality [6], suggesting an essential role of RFX1 in early embryonic development/survival. Knockout of *Rfx* homolog in *Caenorhabditis elegans* and *Drosophila* results in severe sensory defects [7, 8]. Of the mammalian organs and tissues examined, the highest expression level of RFX1 mRNA is in the brain [2]. We have also shown that RFX1 proteins are expressed in the neurons of rat brain and can regulate the expression of the neuronally expressed glutamate transporter type 3 [9]. These results suggest an important role for RFX1 in the central nervous system. However, no detailed study on the RFX1 expression profile in the brain has yet been reported. As an first step to understanding the biological function of RFX1 in mammalian brains, we studied the expression of RFX1

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proteins in the mouse brain. We used the RFX1 mutant mice generated in our previous study [6] to take advantage of the fact that RFX1 expression can be studied not only by routine techniques such as Western blotting, but also by LacZ (β-galactosidase) staining, because those mice have a *β-galactosidase* gene whose expression is under the control of *Rfx1* promoter.

Material and methods

Animals. As previously described [6], RFX1 mutant mice were generated using the gene trap technique with the embryonic stem cell clone RRO347 [*Rfx1*$^{Gt(RRO347)Byg}$]. The gene trap vector was pGT2Lxf that contained a coding region for LacZ and was inserted into the intron sequence between the exon 2 and exon 3. The mice produced were on C57Bl/6J × 129 gene background and were genotyped by polymerase chain reaction (PCR) at the age of 18 days. The *Rfx1*$^{+/-}$ mice were mated and the wild-type *Rfx1*$^{+/+}$ and heterozygous *Rfx1*$^{+/-}$ offspring of the third and fourth generations were used in this study.

Weighing. Four litters of mice including 15 heterozygous *Rfx1*$^{+/-}$ and nine wild-type *Rfx1*$^{+/+}$ mice were weighed at the ages of one, four, seven, 21, 35 and 60 days. The mice were returned to the cages with their mothers immediately after weighing.

Brain sectioning. Ten seven-week old heterozygous *Rfx1*$^{+/-}$ mice were euthanized using isoflurane and transcardially perfused by cold normal saline. For immunofluorescent staining, the mice (five of them) were then perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH = 7.4) and the brains were post-fixed in 4% paraformaldehyde in PBS for 24 hours at 4°C. The brains that were fixed by paraformaldehyde (for immunofluorescent staining) or were not exposed to paraformaldehyde (for β-galactosidase staining, five mice) were immersed sequentially in 10%, 20%, and 30% sucrose-phosphate buffer solution. Finally, 14 μm-thick cryostat coronal sections were obtained for β-galactosidase staining or immunofluorescent staining.

β-galactosidase staining. β-galactosidase staining was performed using the β-Gal Staining Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with minor modifications. Briefly, brain sections were fixed with a fixative solution containing 2% formaldehyde and 0.2% glutaraldehyde in PBS for ten minutes at room temperature. After being rinsed twice, staining solution was added to the sections and incubated for two hours at 37°C, and then at 4°C overnight.

Immunofluorescent staining. Antigen retrieval with microwave heating for 15 minutes in 10 mM tri-sodium citrate buffer (pH = 6.0) containing 0.05% tween-20 was performed as described previously [10, 11]. After being cooled at room temperature, the sections were blocked with 5% donkey serum in PBS containing 0.1% triton-X 100 and 0.05% tween-20. For single- or double-labeled immunofluorescent staining, the following primary antibodies were used: rabbit polyclonal anti-RFX1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-microtubule-associate protein 2 (MAP2) (1:1,000; Abcam, Cambridge, MA, USA); rat monoclonal anti-cluster of differentiation molecule 11b (CD11b) (1:100; Abcam); and mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:600; Millipore, Billerica, MA, USA). After being incubated with the primary antibodies at 4°C overnight, the Cy3-conjugated goat polyclonal anti-rabbit serum (1:100, Abcam) or Alexa Flour 488-labeled goat anti-mouse/rat serum secondary antibody (1:1,000, Invitrogen, Carlsbad, CA, USA) was applied for one hour at room temperature. Negative control sections were incubated with PBS as a substitute for primary antibody.

Western blotting. Each of the seven week-old heterozygous mice was gender-matched with a wild-type mouse from the same litter. These heterozygous *Rfx1*$^{+/-}$ and wild-type *Rfx1*$^{+/+}$ mice were euthanized using isoflurane and transcardially perfused by cold normal saline. Their olfactory bulbs and hippocampi were harvested. Total lysates of these brain regions (50 μg proteins per lane) were subjected to Western analysis as described previously [12]. The primary antibodies used were the rabbit polyclonal anti-RFX1 antibody (1:5,000 dilution, a gift from Dr. Patrick Hearing, State University of New York at Stony Brook, NY, USA) and the rabbit polyclonal anti-actin antibody (1:5,000 dilution; catalog number: A2066; Sigma Chemical, St. Louis, MO, USA). The protein bands were visualized using enhanced chemiluminescence methods. Quantitative analysis of the protein bands was performed using an ImageQuant 5.0 GE Healthcare Densitometer (GE Healthcare, Sunnyvale, CA, USA). The densities of RFX1 protein bands were normalized to those of actin in the same sample to control for errors in protein sample loading and transferring during Western analysis. The result for each mouse was then normalized by the mean values of wild-type mice in the same experiments. To determine the developmental brain expression profile of RFX1, cerebral hemispheres were harvested from wild-type C57Bl/6J mouse (Charles River Laboratories; Wilmington, MA, USA) embryos at embryonic day 15. Hippocampi were also harvested from one week, one month or four month old wild-type C57Bl/6J mice. These brain tissues were subjected to Western blotting to determine RFX1 protein expression.

Real-time PCR. Real-time PCR was performed as described previously [13]. Total RNA was extracted from the olfactory bulbs and hippocampi of mice aged seven weeks using an RNeasy micro kit (Qiagen, Valencia, CA, USA).

As with Western blotting, littermates of the heterozygous and wild-type mice that were gender-matched were used in this study. Primers for real-time PCR were designed based on the reported sequence of mouse *Rfx1* gene using the Primer Express 3.0 software (Applied Biosystems, Carlsbad, CA, USA) and selected to best fit the requirement of SYBR Green assays. The sequences of the primers are: forward, AGTGAGGCTCCACCACTGGCCG and reverse, TGGGCAGCCGCTTCTC. The sequences corresponding to these two primers in the *Rfx1* gene are in exon 14 and exon 15, respectively. Quantitative PCRs were carried out in triplicate using each cDNA sample that was equivalent to 50 ng of starting total RNA. Amplifying PCR and monitoring of the fluorescent emission in real-time were performed in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). To verify that only a single PCR product was amplified per transcript, dissociation curve data was analyzed using 7900HT Sequence Detection software. To account for possible differences in starting material, quantitative PCR of the housekeeping genes glyceraldehydes-3-phosphate dehydrogenase and actin was also carried out for each cDNA sample. The relative amount of RFX1 mRNA in each sample was determined using the comparative threshold cycle method and then normalized to those of housekeeping genes.

Statistical analysis. Western blotting and real-time PCR results were presented as means ± SD and analyzed by *t*-test. A $p \geq 0.05$ was accepted as significant.

Results

The heterozygous *Rfx1*$^{+/-}$ mice appeared normal. Their growth curve overlapped with that of the wild-type mice (Figure 1), suggesting that there was no growth retardation in the heterozygous *Rfx1*$^{+/-}$ mice.

Since the β-galactosidase expression in the heterozygous *Rfx1*$^{+/-}$ mice is under the control of the *Rfx1* promoter, the expression of β-galactosidase reflects the specific expression of RFX1. Brain sections from each of the five heterozygous *Rfx1*$^{+/-}$ mice that were used to cut sections all were β-galactosidase staining positive. β-galactosidase staining was prominent in the olfactory bulb, cerebral cortex and hippocampus. A significant amount of β-galactosidase staining also appeared in the striatum (Figure 2). These brain regions were positively stained by immunofluorescent cytochemistry with the anti-RFX1 antibody (Figure 3).

To identify cell-type origin of RFX1 proteins, we used double-labeled immunofluorescent staining. As shown in Figure 4, the RFX1 positive staining is co-localized with the staining of MAP2, a neuronal specific protein, and CD11b, a microglial cell-specific protein. These results suggest that RFX1 is expressed

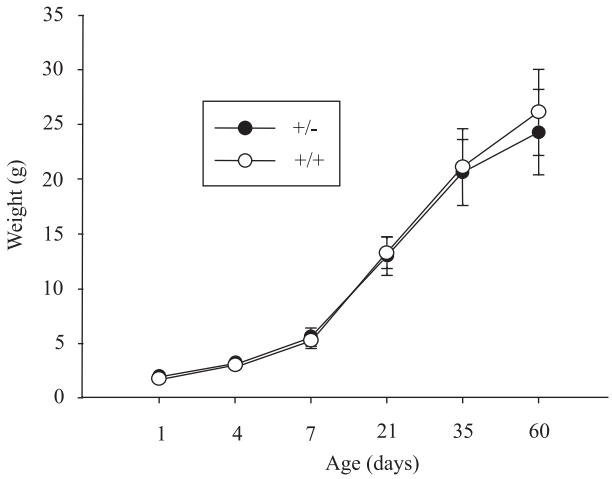

Figure 1. Weights of the heterozygous *Rfx1*$^{+/-}$ and wild-type littermates. Results are means ± SD (n = 15 for the heterozygous [ten females/five males] and n = 9 for the wild-type mice [five females/four males])

in the neurons and microglial cells. The RFX1 positive staining was not co-localized with GFAP, an astrocyte-specific protein. In addition, the RFX1 positive staining mainly appeared in the nuclei of neurons and microglial cells (Figure 4).

Quantitative determination of RFX1 expression was performed in the olfactory bulb and hippocampus because these two brain structures expressed a significant amount of RFX1 proteins, as described above. Also, these two structures represent functional diversity of the brain: the olfactory bulb is involved in the sensory neurotransmission of smell, and the hippocampus plays an important role in learning and memory. There was no statistically significant difference in RFX1 mRNA and protein expression in the olfactory bulbs and hippocampi between the seven week old heterozygous *Rfx1*$^{+/-}$ mice and the wild-type mice (Figure 5). There was also no evidence of gender difference in RFX1 protein expression. For example, the relative RFX1 protein levels after being normalized by the results of actin were 1.02 ± 0.26 and 0.98 ± 0.22 (arbitrary unit), respectively, in the hippocampi of male and female wild-type mice aged seven weeks ($p = 0.827$, $n = 4$). In addition, there was no significant change in RFX1 protein expression in the brain tissues of the wild-type C57Bl/6J mice that ranged from 15 day old embryos to four month old adult mice (Figure 6), suggesting that RFX1 is maintained at a stable level throughout the period from late embryonic stage to adulthood.

Discussion

The mouse *Rfx1* gene has 21 exons that produce a protein containing 963 amino acids [14]. The inser-

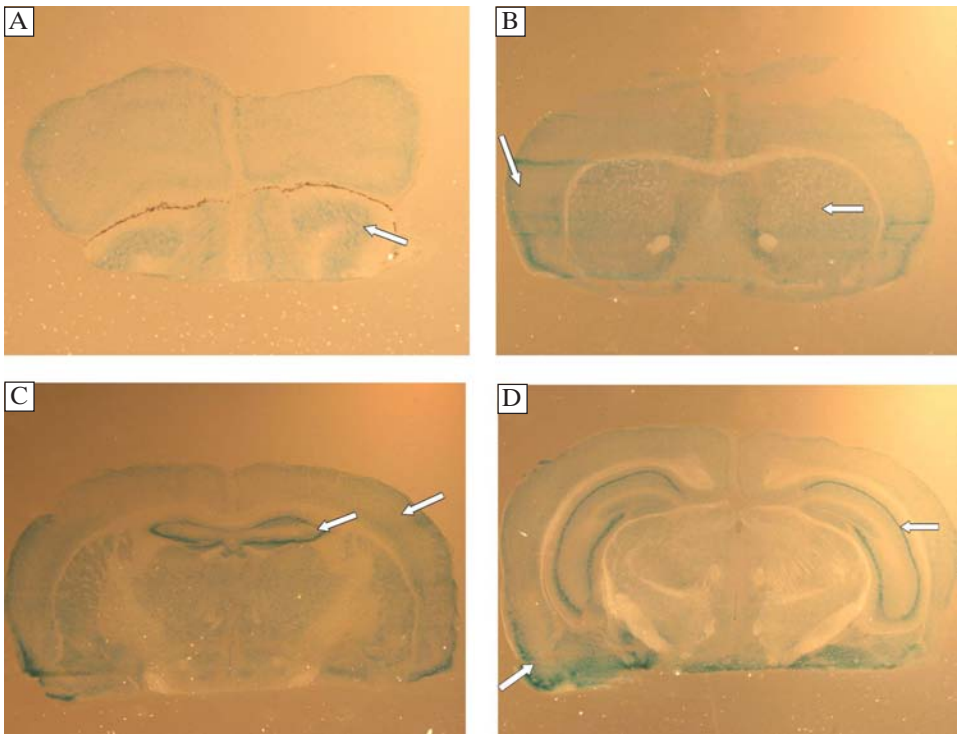

Figure 2. β-galactosidase staining of the heterozygous *Rfx1*$^{+/-}$ mouse brain sections. The brain sections are arranged from rostrum to caudum in the order of panel **A** to panel **D.** Positive staining of β-galactosidase is blue and is pointed by an arrow. The arrow in panel **A** indicates anterior olfactory nucleus. The arrows in panel **B** indicate cerebral cortex and caudate putamen, respectively. The arrows in panels **C** and **D** indicate cerebral cortex and hippocampus, respectively

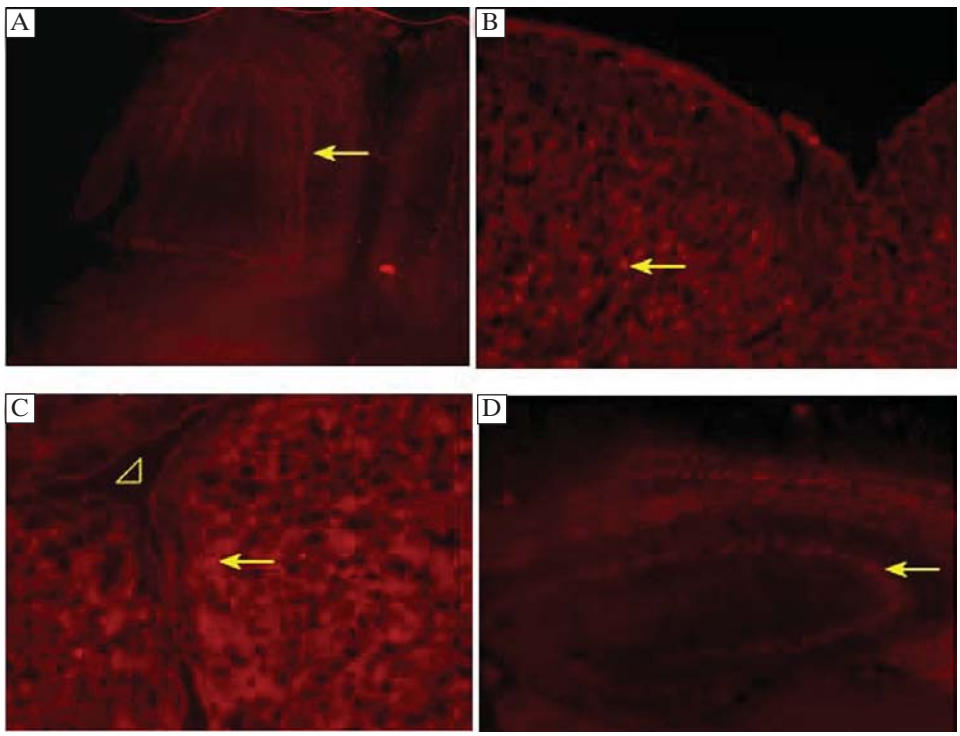

Figure 3. Immunofluorescent staining of heterozygous *Rfx1*$^{+/-}$ mouse brain sections with an anti-RFX1 antibody. Positive staining, in red fluorescence, is shown in the olfactory bulb (**A**), striatum (**B**), cerebral cortex (**C**) and hippocampus (**D**), and is pointed by an arrow. A triangle in panel **B** indicates a lateral ventricle

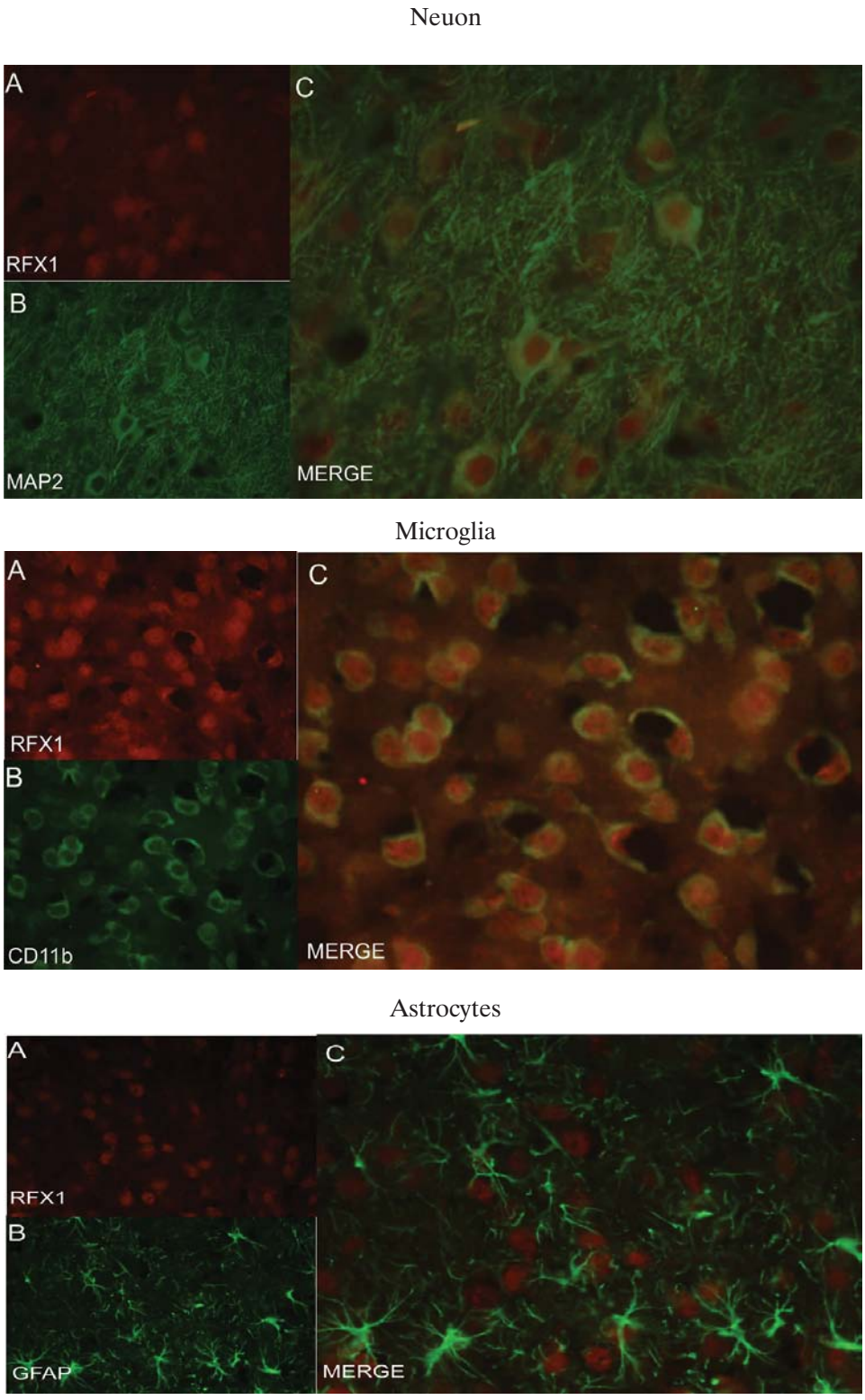

Figure 4. Immunofluorescent staining of heterozygous *Rfx1*$^{+/-}$ mouse brain sections. Photographs were taken from cerebral cortex. RFX1 positive staining is in red fluorescence. Positive immunofluorescent staining for MAP2 (top panel), CD11b (middle panel) and GFAP (bottom panel) is in green fluorescence. There are three small panels in each large panel. Panel **A** shows positive staining for RFX1 proteins; panel **B** shows positive staining for MAP2, CD11b or GFAP, respectively; and panel **C** is the merged images of panel **A** and panel **B**

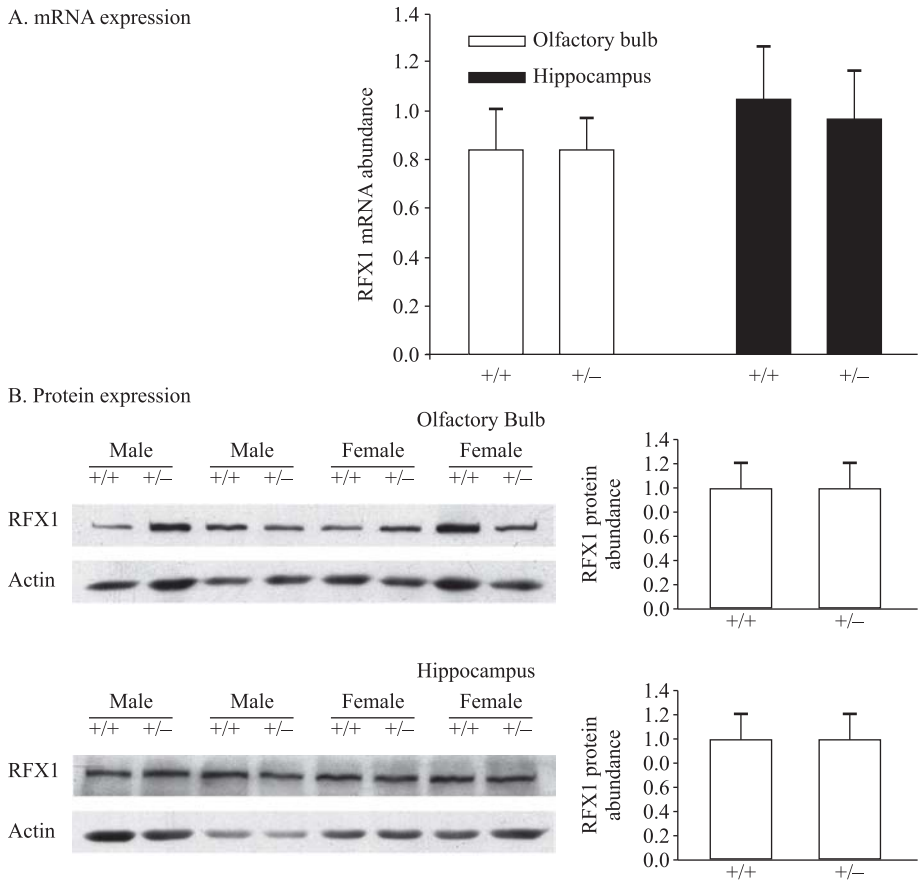

Figure 5. RFX1 mRNA and protein expression in the heterozygous *Rfx1*$^{+/-}$ and wild-type littermates. Each heterozygous *Rfx1*$^{+/-}$ mouse was gender-matched with one wild-type mouse from the same litter. Their olfactory bulbs and hippocampi were isolated for real-time polymerase chain reaction and Western blotting. The relative RFX1 mRNA and protein abundance after being normalized by the results of actin in the same sample is presented in panel **A** and panel **B**, respectively. The protein results were further normalized by the mean values of wild-type littermates in the same Western blot. In panel **B**, a representative Western blot is presented in the left panels and the pooled results are presented as a bar graph in the right panels. Results are means ± SD (n = 8 for each bar in the pooled results)

tion of the gene trap vector in the embryonic stem cell clone RRO347A that was used to generate our mutant mice is in the intron between the exon 2 and exon 3. This insertion can result in production of a truncated protein that contains the first 97 amino acids of the RFX1 protein. Function domains, such as DNA binding and dimerization domains, that are important for RFX1 to function as a transcription factor are in the segments that start after the first 140 amino acids in the molecule [14]. Thus, the truncated proteins produced in our mutant mice should not have the functions of RFX1. Consistent with this loss-of--function concept, *Rfx1* knockout leads to embryonic lethality before the age of 3.5 days [6].

Currently, RFX1 functions are largely unknown. A few studies have indicated a role of RFX1 in the central nervous system [7–9]. However, there has only been one study describing the distribution of RFX1 proteins in mammalian brains [9]. It showed by immunocytochemistry that RFX1 proteins were in the neurons of the cerebral cortex, hippocampus and cerebellum of rats [9]. However, the study did not include detailed investigation of the brain region distribution and cell type origin of RFX1 proteins. Our current study suggests that olfactory bulb and striatum also express RFX1 protein in the mouse. In addition to neurons, RFX1 proteins are expressed in microglial cells. Consistent with the nature of transcription factors, the RFX1 proteins were mainly expressed in the nuclei. Interestingly, RFX1 expression in the brain does not change significantly from E15 to adult mice. RFX1 has been shown to regulate expression of early response genes and growth factors [15, 16]. Our recent study showing embryonic lethality of *Rfx1* knockout suggests a critical role of RFX1 in early embryonic development and cell survival [6]. In addition, we have shown that RFX1 regulates the expression of glutamate transporter type 3 [9], a neuron-specific glutamate transporter that is involved in regulating glutamate neurotransmission and learning

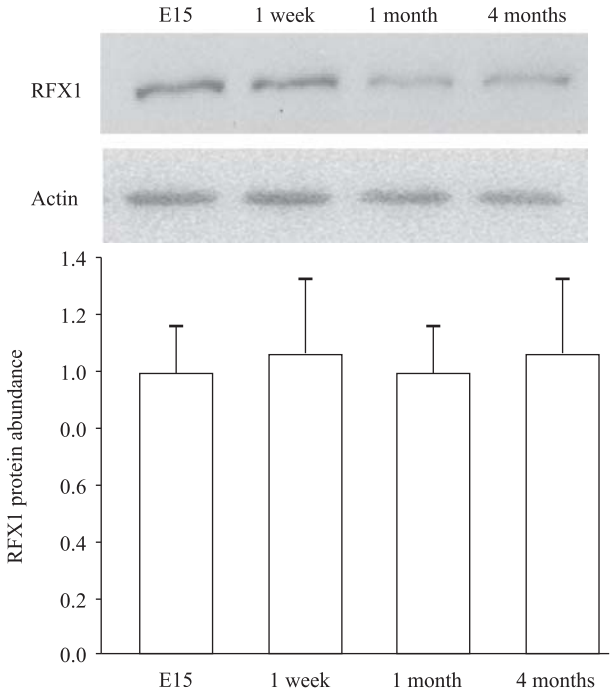

Figure 6. RFX1 protein expression in the wild-type C57Bl/6J mice. Cerebral hemispheres were harvested from embryos at embryonic day 15 (E15). Hippocampi also were harvested from one week, one month or four month old wild-type mice. They then were subjected to Western blotting. A representative Western blot is presented in the top panels and the pooled results of the relative RFX1 protein abundance after being normalized by the results of actin in the same sample, and then the mean values of E15 embryos in the same blot are presented as a bar graph in the bottom panels. Results are means ± SD (n = 6 for each bar in the pooled results)

and memory functions [17, 18]. These findings suggest a broad range of functions of RFX1 in neurons. This suggestion is supported by a ubiquitous expression pattern of RFX1 in the brain as shown in this study.

A common rule that is generally assumed to control gene expression in diploid eukaryotic organisms is that maternally and paternally derived copies of each gene are simultaneously expressed at similar levels. However, it is well-known that there is a monoallelic expression pattern in which only one of the two alleles is expressed. Our results suggest that *Rfx1* gene takes a monoallelic expression pattern in the brain because the heterozygous *Rfx1*$^{+/-}$ mice had a similar level of RFX1 mRNA and protein to that of the wild-type mice in the olfactory bulbs and hippocampi.

There are three classes of monoallelic expression patterns [19].

The first class includes X-inactivated genes. Regulation of RFX1 expression does not belong to this class because RFX1 gene is in chromosome 19 in humans and chromosome 8 in mice [20].

The second class consists of autosomal imprinted genes whose expression is controlled in a parent-of-origin-specific pattern [21]. Our previous study showed that knockout of *Rfx1* gene expression leads to early embryonic death and the ratio of the wild-type mice to the heterozygous *Rfx1*$^{+/-}$ mice in the living mice of our large sample (154 mice) was about 1:2 [6]. If *Rfx1* expression takes the form of imprinting to regulate its expression, and this reprinting occurs very early in the embryonic development, we would expect a 1:1 ratio of the living heterozygous *Rfx1*$^{+/-}$ mice to the wild-type mice. If this reprinting happens later, we would expect that some of the heterozygous *Rfx1*$^{+/-}$ mice do not express RFX1 proteins. We examined RFX1 protein expression in the hippocampus and olfactory bulb by Western blotting. All of the heterozygous *Rfx1*$^{+/-}$ mice expressed RFX1 in these two brain regions, and all five heterozygous Rfx1$^{+/-}$ mice whose brains were sectioned were β-galactosidase staining positive. Thus, RFX1 expression may not be regulated by reprinting in the brain.

The third and final class of monoallelic expression is the random monoallelic expression of autosomal genes [19]. This group of genes takes a random form to control their gene expression: some cells express the maternal allele, and other cells express the paternal allele. In some cases/genes, cells express both alleles. The heterozygous *Rfx1*$^{+/-}$ mice and the wild-type mice expressed a similar level of RFX1 mRNA and proteins in the hippocampus and olfactory bulb, and the brain regional distribution of RFX1 proteins and β-galactosidase is very similar in the brains of the heterozygous *Rfx1*$^{+/-}$ mice. Thus, RFX1 expression may take the form of random monoallelic expression in which cells express products from both alleles. However, our results cannot completely exclude the possibility that RFX1 expression takes a biallelic pattern. The evidence to support this pattern is our finding that both alleles are expressed. Although a similar level of RFX1 in the heterozygous *Rfx1*$^{+/-}$ and wild-type littermates does not support the biallelic expression pattern, this finding could be due to the results of compensatory mechanisms to maintain a critical level of RFX1 in the heterozygous *Rfx1*$^{+/-}$ mice.

We have observed that the heterozygous *Rfx1*$^{+/-}$ mice appeared normal and did not have growth retardation. These findings are consistent with our results that the heterozygous *Rfx1*$^{+/-}$ mice did not have a significant change in the RFX1 protein expression in their brains.

In summary, we have shown that RFX1 proteins are abundantly expressed in the hippocampus, cerebral cortex and olfactory bulb, and are also expressed in the striatum. The heterozygous *Rfx1*$^{+/-}$ mice have

a similar level of RFX1 mRNA and proteins to that of the wild-type mice in the olfactory bulbs and hippocampi. These proteins are mainly expressed in the nuclei of neurons and microglial cells. These results, along with our previous findings that RFX1 is critical for embryonic development/survival and that the ratio of the living heterozygous *Rfx1*$^{+/-}$ mice to the wild-type mice is about 2:1 [6], suggest that *Rfx1* takes the form of random monoallelic expression pattern to regulate its expression.

Conflict of interest

The authors declare that they have no competing financial or other conflict of interest.

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