Loss of aquaporin-4 impairs cerebrospinal fluid solute clearance through cerebrospinal fluid drainage pathways

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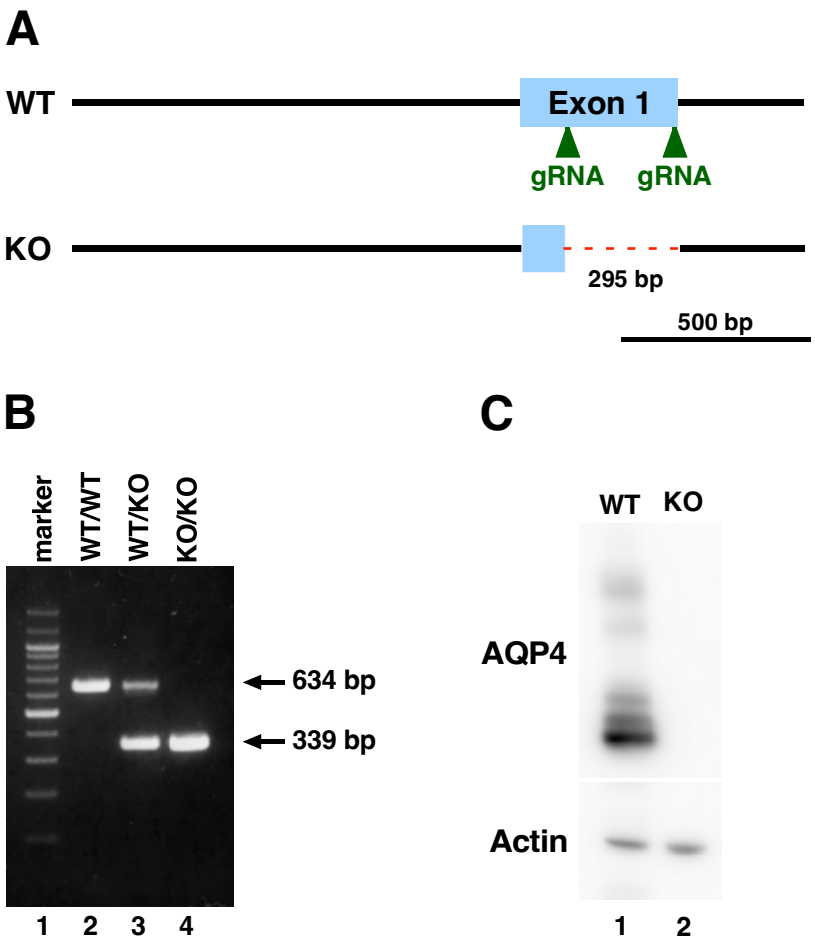
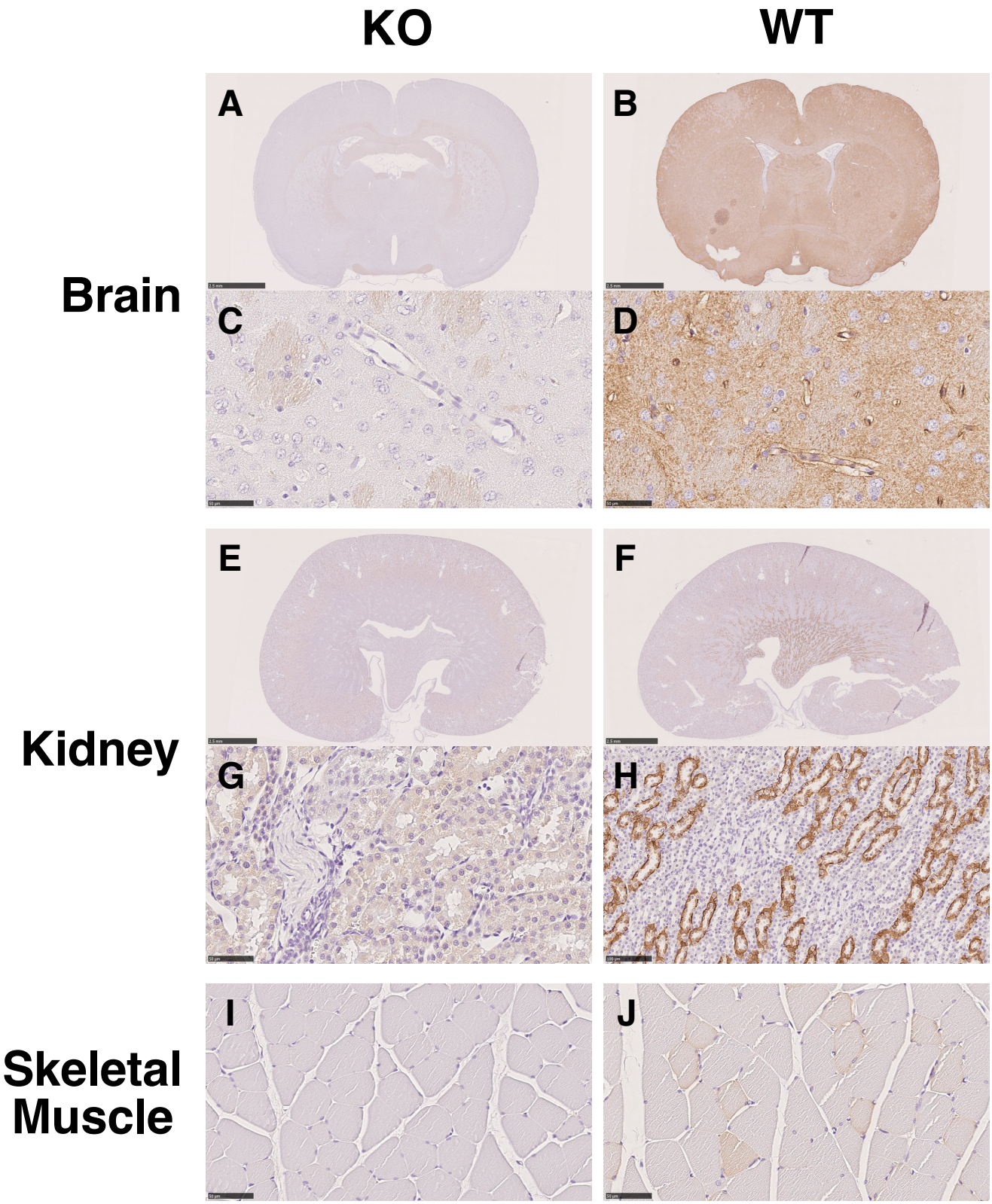
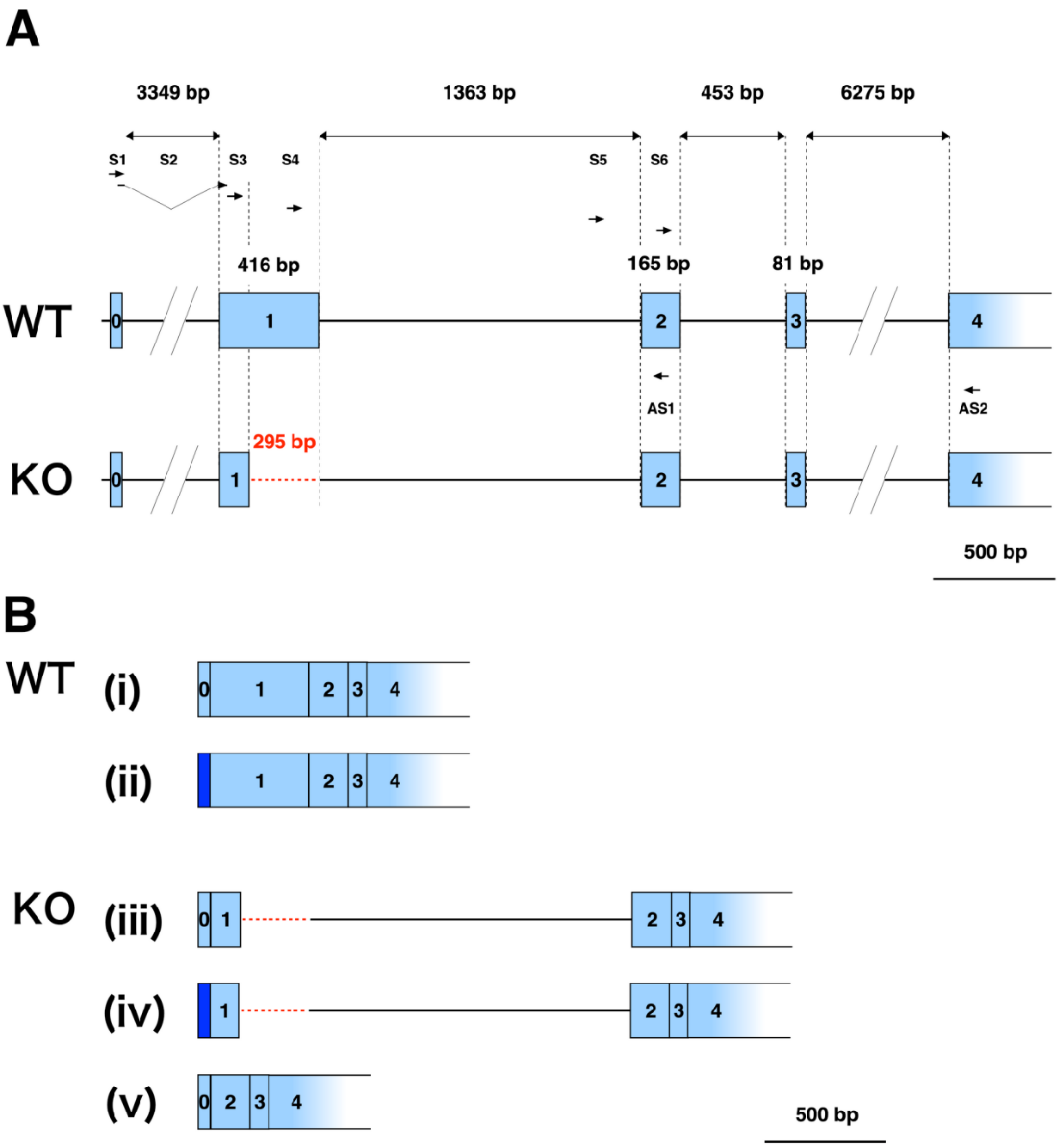


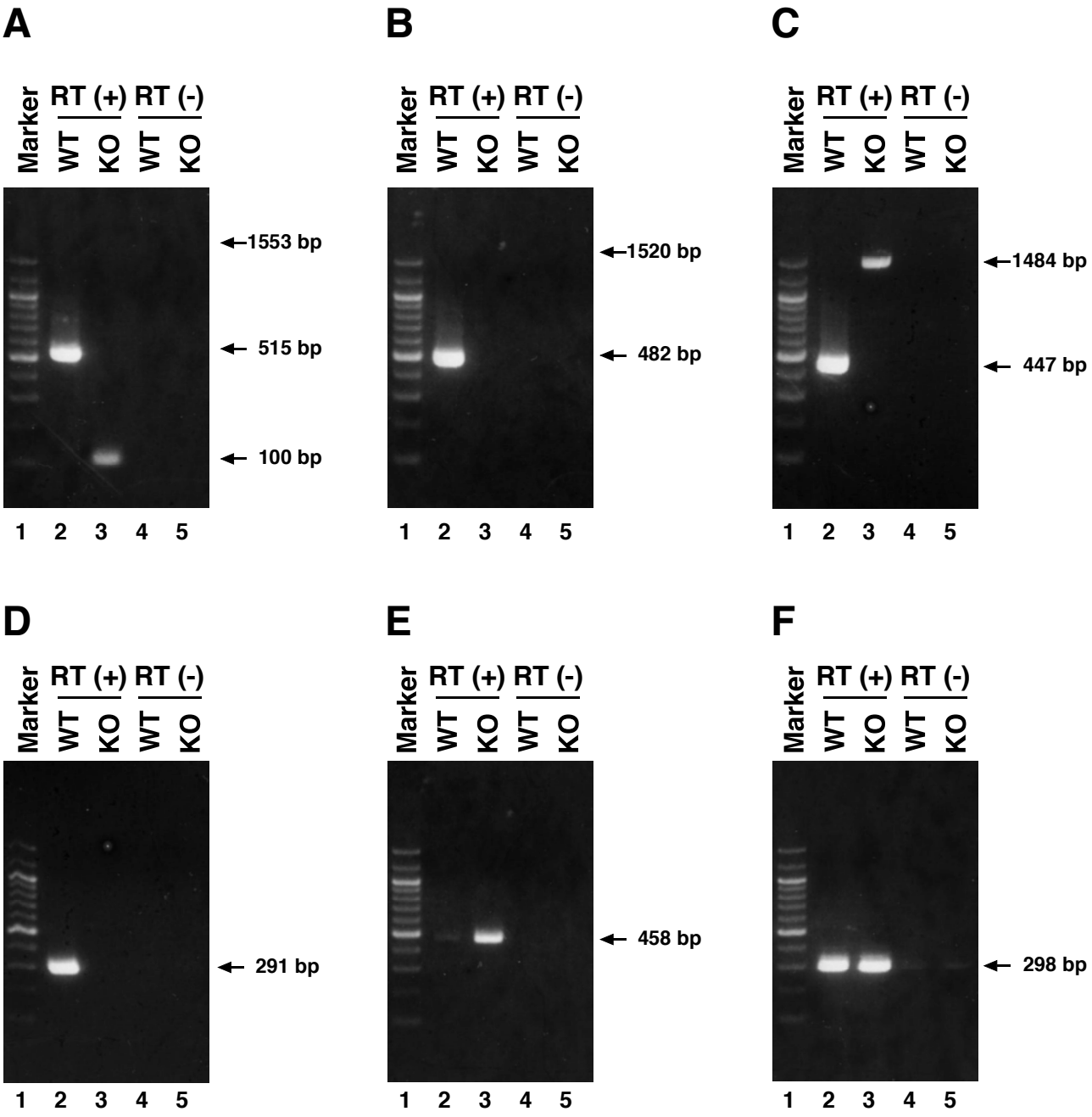
Supplemental Figure S1. Establishment of AQP4 KO rats.
(**A**) A schematic illustration depicting the genome editing strategy and the resulting genomic structure of AQP4 KO rats. An exon is represented by a blue box. A deleted part is represented by a red dotted line. (**B**) A method of genotyping for the AQP4 KO rats using PCR. Single 634-bp and 339-bp bands were amplified from genomic DNA extracting from homozygous wild-type (lane 2) and KO (lane 4) rats, respectively, and both bands were observed in heterozygotes (lane 3). A pattern of 100-bp ladder is also shown (lane 1) (**C**) Expression of AQP4 (upper panel) and actin (lower panel) in the cerebellum of wild-type (lane 1) and AQP4 KO rats (lane 2) determined by Western blotting. The original blots are presented in Supplemental Figure S6.



Supplemental Figure S2. Immunohistochemical analysis of AQP4 KO rats.
Paraffin-embedded sections of brains (**A-D**), kidneys (**E-H**) and skeletal muscles (**I, J**) of KO (**A, C, E, G, and I**) and wild-type (**B, D, F, H, and J**) rats were stained by anti-AQP4 antibody. Bars = 2.5 mm (**A, B, E, and F**) or 50 μm (**C, D, G, H, I, and J**)



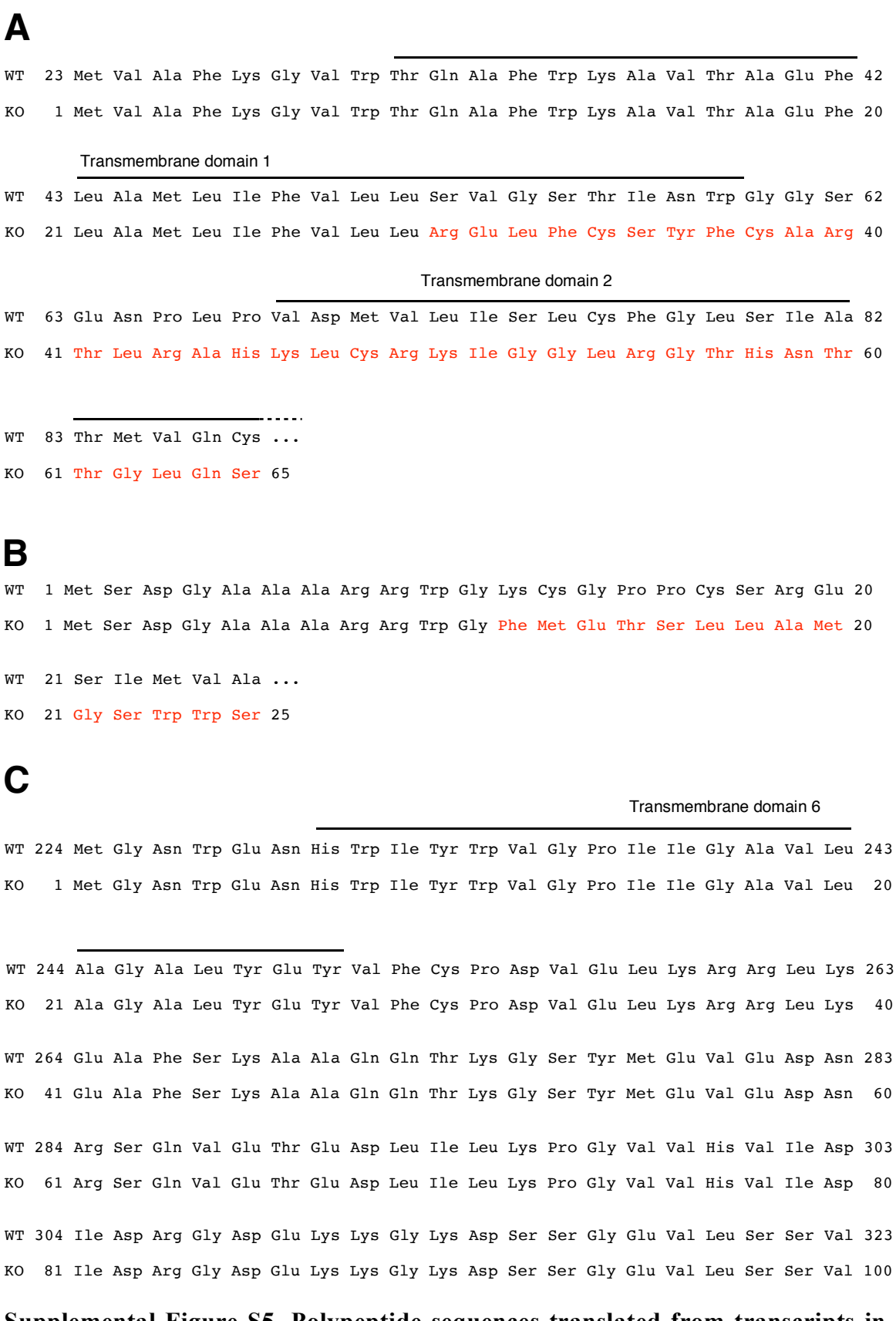
Supplemental Figure S3. Analysis of the structure of transcrtipts expressed in AQP4 KO rats. (**A**) A schematic illustration that compares the structure of the genomic DNA of AQP4 KO rats with that of wild type. Exons are represented by blue boxes. A 295-bp deleted part in the genomic DNA of KO rats is represented by a red dotted line. Primers used for PCR are represented as arrows. Their sequences are shown in Supplemental Table 1. Sizes of possible transcripts are shown in Supplemental Table 2. (**B**) Schematic illustrations of structure of transcripts possibly expressed in KO rats (iii - v) comparing with that of wild type (i and ii). Since the knockout allele lacks a 5'-splice site at the boundary of exon 1 and flanking 3'-intron, it is possible that the remaining part of exon 1, the following intron, and exon 2 as a large exon (iii and iv) or the remaining part of exon 1 and its flanking introns are recognized as an intron (v).



Supplemental Figure S4. Detection of transcripts expressed in the cerebellum of AQP4 KO rats by RT-PCR.

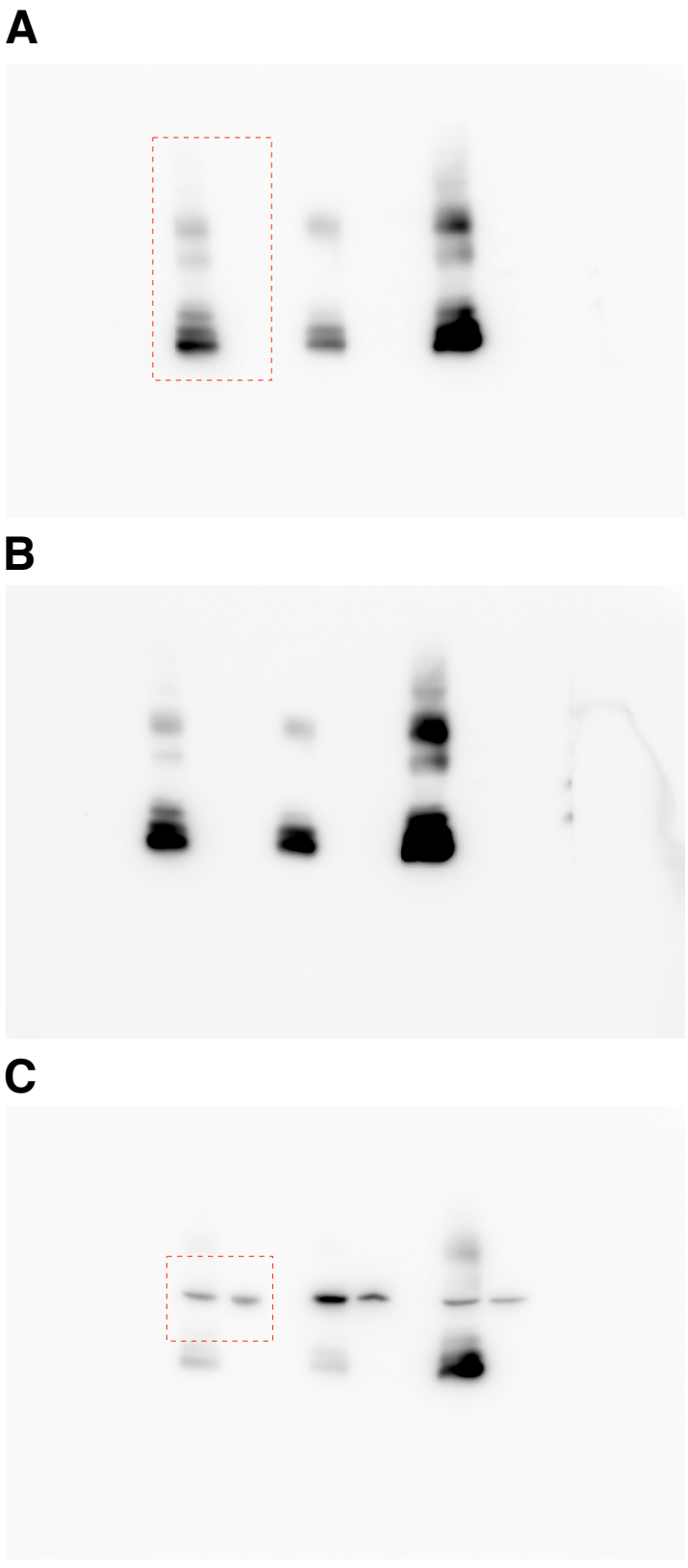
Total RNA extracted from cerebella of wild type (lanes 2 and 3) and knockout (lanes 3 and 5) rats were subjected to reverse transcription reaction in the presence (lanes 1 and 2) or absence (lanes 4 and 5) of reverse transcriptase followed by PCR reaction using primer sets S1-AS1 (**A**), S2-AS2 (**B**), S3-AS1 (**C**), S4-AS1 (**D**), S5-AS2 (**E**) and S6-AS2 (**F**) (Supplemental Figure S3). No amplification was observed when reverse transcriptase was absent in the reaction mixtures (lanes 4 and 5), confirming that no amplification derived from genomic DNA was detected. A pattern of 100-bp ladder is also shown (lane 1).

(**A**) A shorter (100 bp) fragment was amplified in KO by primer set S1-AS1. Cloning and sequence of this fragment revealed that in KO rats, there exists a transcript in which exon 0 is directly spliced to exon 2. On the other hand, this primer set did not amplify a 1553-bp band, indicating lack of transcript iii shown in Supplemental Figure S3B. (**B**) No band was detected in KO rats by primer S2, which recognizes the junction between exons 0 and 1, indicating that there is no transcript in which exon 0 is conected to exon 1, confirming the lack of transcript iii. (**C**) A larger band (1484 bp) was amplified in KO by primer set S3-AS1, indicating that there is an insertion between the remaining portion of exon 1 and exon 2. (**D**) No band was detected in KO rats by primer S4, which recognizes a sequence deleted in KO rats. (**E**) A 458-bp band was amplified in KO rats by primer set S5-AS2, confirming that a transcript in KO rats includes an intron flanked by exon 2. (**F**) A significant level of the band amplified by primer set S6-AS2, which amplifies between exons 2 and 4, was observed in KO rats. Taken together, it is highly likely that transcripts iv and v are dominantly expressed in AQP4 KO rats.



Supplemental Figure S5. Polypeptide sequences translated from transcripts in AQP4 KO rats.

(**A**) A polypeptide encoded by transcript iv in Supplemental Figure S3B. (**B**) A polypeptide encoded by transcript v in Supplemental Figure S3B. (**C**) Since the initiation codon for the M1 isoform located in exon 0 is not perfectly match for the Kozak consensus sequence and therefore, the 224th methionine could alternatively function as an initiation codon encoding a 100-bp truncated AQP4 having the sixth transmembrane and the C-terminal domains. Amino acid sequences unrelated to AQP4 caused by truncation of exon 1 or unusual splicing in KO rats are indicated in red. Transmembrane domains of wild-type AQP4 are indicated with lines according to Hiroaki et al., (2006).



Supplemental Figure S6. The original blots for Supplemental Figure S1C
(**A**) The original blot used for Supplemental Figure S1C using anti-AQP4 C-terminal domain followed by HRP-labeled anti-rabbit IgG. The cropped area produced using Canvas X Draw ver. 7.0.4 (Canvas GFX Inc., Boston, MA) is indicated by red dotted line. (**B**) Longer exposure of the same membrane presented in (**A**) is shown in order to make membrane edge visible. (**C**) The same membrane used in (**A**) was re-probed with HRP-labeled anti-actin antibody. The cropped area is indicated by red dotted line.

Supplemental Table 1. The list of primers used for determination of the structure of AQP4 transcripts.

ID	*Sequence*
S 1	5′-ATGAGAGCTGCACTCCGGCCAGGGAAGGCATGAG-3′
S 2	5′-AGTGACGGAGCTGCAGCGAGGCGGTGGGGTAAGTG-3′
S 3	5′-GGACCTCCCTGCAGCAGAGAGAGCATCATGGTGGC-3′
S 4	5′-CTACCTGTGGACATGGTCCTCATCTCCCTCTGC-3′
S 5	5′-GTGGAAGGCATGGAAATCTCTGTGTGGGGTTGGTG-3′
S 6	5′-GAAACCTCACTGCTGGCCATGGGCTCCTGGTG-3′
AS 1	5′-CACCAGGAGCCCATGGCCAGCAGTGAGGTTTC-3′
AS 2	5′-ACATACTCGTAAAGTGCACCTGCCAGCACAGC-3′

Supplemental Table 2. Sizes of RT-PCR products amplified from each predicted transcript.

Transcripts	*Primer Sets*					
	S1-AS1	**S2-AS1**	**S3-AS1**	**S4-AS1**	**S5-AS2**	**S6-AS2**
(i)	**515 bp**	**482 bp**	**447 bp**	**291 bp**		**298 bp**
(ii)			**447 bp**	**291 bp**		**298 bp**
(iii)	**1553 bp**	**1520 bp**	**1484 bp**		**458 bp**	**298 bp**
(iv)			**1484 bp**		**458 bp**	**298 bp**
(v)	**100 bp**					**298 bp**