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Molecules and Cells

Blood-Brain Barrier Defects Associated with *Rbp9* Mutation

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***Rbp9* is a *Drosophila* RNA-binding protein that shares a high level of sequence similarity with *Drosophila elav* and human Hu proteins. Loss of function alleles of *elav* are embryonic lethal causing abnormal central nervous system (CNS) development, and Hu is implicated in the development of paraneoplastic neurological syndrome associated with small cell lung cancer. To elucidate the role of *Rbp9*, we generated *Rbp9* mutant flies and examined them for symptoms related to paraneoplastic encephalomyelitis. Although Rbp9 proteins begin to appear from the middle of the pupal period in the cortex of the CNS, the *Rbp9* mutants showed no apparent defects in development. However, as the mutant adult flies grew older, they showed reduced locomotor activities and lived only one-half of the life expectancy of wild-type flies. To understand the molecular mechanism underlying this symptom, gene expression profiles in *Rbp9* mutants were analyzed and potential target genes were further characterized. Reduced expression of cell adhesion molecules was detected, and defects in the blood-brain barrier (BBB) of *Rbp9* mutant brains could be seen. Putative *Rbp9*-binding sites were found in introns of genes that function in cell adhesion. Therefore, *Rbp9* may regulate the splicing of cell adhesion molecules, critical for the formation of the BBB.**

INTRODUCTION

Rbp9 was initially cloned during the process of identifying RNA binding protein-coding genes based on its sequence characteristics as possessing RNA recognition motifs (Kim and Baker, 1993). *Rbp9* is expressed in postmitotic cells of both neuronal and female ovarian germ line origins (Kim-Ha et al., 1999). *Rbp9* mutants showed no obvious developmental or behavioral defects during development until the young adult stage. Mutational analysis revealed that *Rbp9* is required for cystocyte development and lack of *Rbp9* results in an ovarian tumor phenotype (Kim-Ha et al., 1999). The role of *Rbp9* in the regulation of cell proliferation has been further assessed in female germ cells; the precocious expression of *Rbp9* was found to inhibit ovarian germ cell proliferation (Jeong and Kim-Ha, 2004).

The Rbp9 protein is highly homologous to the *Drosophila elav* and human Hu proteins. *Elav* is a pan-neuronal gene whose absence causes embryonic lethality due to defects in nervous system development (Robinow and White, 1991). The molecular function of *elav* was shown to promote the formation of the neural-specific alternative splice variant of *Neuroglian* (Koushika et al., 1996) and increase splicing efficiency of *erect wing* transcripts in alternatively spliced regions (Koushika et al., 2000). Hu proteins also contain RNA-binding motifs and are involved in many biological processes. One of the phenotypes associated with the Hu protein is paraneoplastic neurologic disorder (PND). PND is characterized by degeneration of the nervous system and the presence of cancer in the body (Honnorat and Cartalat-Carel, 2004). One hundred percent of small cell lung cancer (SCLC) patients exhibiting the typical paraneoplastic neurological syndrome develop anti-Hu antibodies. As anti-Hu antibodies recognize Hu antigens expressed by SCLC and neurons, both regression of SCLC and progression of neurological symptoms occur simultaneously.

As *Rbp9*, *elav* and Hu are all expressed in brain and both *elav* and Hu were characterized to possess neuronal function, we examined *Rbp9* mutant flies for symptoms related to neurological defects. During early development, *Rbp9* mutant flies did not show signs of any defects but as the mutant adult flies grew older, both male and female flies showed reduced locomotor activities and lived only one-half of the life expectancy of wild-type adults. To elucidate the molecular mechanism underlying this symptom, we examined gene expression profiles using microarrays. From data obtained from microarray analysis and observation of mutant brain morphologies, we confirmed defects in cell adhesion, which lead to defects in blood-brain barrier (BBB) formation and maintenance.

MATERIALS AND METHODS

Measurement of climbing activity, survival and heat sensitivity

Flies were grown on standard *Drosophila* medium at 25°C until eclosion. Age-synchronized flies were obtained by collecting newly emerging flies in a 12-h time window. The flies were placed in fresh vials at 25°C for 1 day. For the climbing and survival test, subsequent cultures were maintained at 29°C and the flies were transferred to new vials with fresh food every day.

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The number of flies that climbed to the top of the vial within 20 s after application of negative geotactic pressure was counted. To test heat sensitivity, the vial containing flies was placed in a 37°C water bath. The number of the flies that retained their climbing ability was measured at 5-minute intervals.

cDNA microarray hybridization

To isolate adult fly heads, whole flies were chilled with liquid nitrogen and vortexed rapidly. Heads were separated using sieves with 710- and 300-μm diameter openings. Total RNA was prepared using TRIZol reagent (Invitrogen). The integrity of the RNA was assessed by electrophoresis with a 2100 Bioanalyzer (Agilent). The cDNA microarrays containing 5,929 cDNA elements (Digital Genomics, Korea) were used for gene expression analysis. The microarrays contained duplicated spots, and data were averaged from duplicated regions. All procedures for cDNA microarray hybridization were previously described by Kim et al. (2005). Probes prepared from males were applied to microarray chips with dye swapping. Probes from females were tested once. Genes with at least a 1.5-fold difference in expression level were selected from each experiment. Candidate genes were selected only if altered in all three experiments.

RT-PCR analysis

From 5 μg total RNA prepared from adult fly heads of both wild-type and *Rbp9* mutant strains, single stranded cDNA was synthesized with oligo dT primers and the final volume was adjusted with distilled water to 200 μl at the end of the reaction. Five microliter cDNA was used for PCR. Primers used were takeout (5′ CAGGGCAAGGTACTCATTCTACCG 3′/5′ CCAGGTAGGTCTCCCCATTCTTCA 3′), CG14521 (5′ CGAGGGCACATTGCGGTTATACG 3′/5′ GTTCTCAGACTCAGCCTCATCC 3′), *Neurexin IV* (5′ GTTATGCCTCCTATTGCTGGTC 3′/5′ CGTTTAGTCGTGCTTTGTCGGG 3′), *Gliotactin* (5′ CATAGTCCTGGTGGTCATCTGC 3′/5′ CCGGTTCCAAGCCCTCACCA 3′), *Neuroglian* (5′ GATGGACAACCCGAGCCAGA 3′/5′ GGTGGCCGTTCCGAATTCA 3′), *coracle* (5′ CTGCCCTAGCACGAGTCACC 3′/5′ CTGTGGGCGTCTCATAGGTCAG 3′), *Neurotactin* (5′ CAAGGACCAACGGATTATTCCC 3′/5′ CTTCTCCTCGAGTTCGCCCAT 3′), *armadillo* (5′ CGGGCACTCATTCGCCAGCA 3′/5′ TCCTTGTCAGCGGCGAGCTCAC 3′) and *elav* (5′ GCGCGCGCAAGGATTTGAGATT 3′/5′ CTCTGCATTAGCTGTGCCTGTG 3′).

Dye penetration assay

For dye injection into adult flies and subsequent assay, we followed the protocol described by Bainton et al. (2005). One hundred and fifty nanoliters of tetramethylrhodamine conjugated dextran (50 mg/ml in PBS, MW 10,000, Molecular Probes) were injected into the abdomen of each wild-type or mutant fly. At 18 or 40 h after injection, brains were dissected, and two brains were placed in each well of a 96-well plate (Corning Costar special optics) containing 50 μl of 0.1% SDS solution. Fluorescence was measured using a SpectraMax Gemini EM microplate spectrofluorometer (Molecular Devices).

RESULTS

***Rbp9* mutant flies prematurely lose climbing ability**

Neurological defects in adult flies often result in premature loss of climbing ability (Feany and Bender, 2000). To gain insight into *Rbp9* function in the nervous system, we examined *Rbp9* mutant flies for their ability to respond to geotactic pressure. *Rbp9* mutant flies initially climbed as well as wild-type flies. However, overtime, *Rbp9* mutant flies declined in performance more rapidly than wild-type flies. Among 13-day-old *Rbp9* mutant flies, 50% lost their climbing ability whereas most of the wild-type flies at same age retained climbing ability (Fig. 1A). The locomotor dysfunction observed in the *Rbp9* mutants was completely rescued by germ-line transformation of a DNA fragment that encompassed the *Rbp9* gene, which we previously reported to rescue the female sterile phenotype of *Rbp9* mutants (Kim-Ha et al., 1999). The longevity of *Rbp9* mutant flies showed a correlation to the severity of the climbing defect. *Rbp9* mutants lived only one-half of the life expectancy of wild-type flies (Fig. 1B).

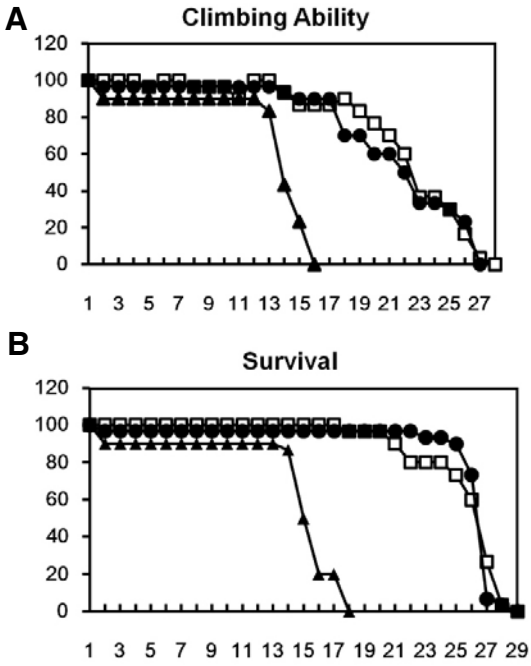

Fig. 1. Premature loss of climbing ability in *Rbp9* mutant flies. (A) *Rbp9* mutant flies (closed trianlges) initially climb as well as wild-type flies (open squares). However, over time, *Rbp9* mutant flies decline in performance more rapidly than wild-type flies. The locomotor dysfunction observed in $Rbp9^{P[2690]}$ is rescued completely by the germ line transformation of a DNA fragment that encompasses the *Rbp9* gene (closed circles). (B) The longevity of *Rbp9* mutant, wild-type- and germ-line-transformed flies showed correlation to the severity of the climbing defect. Flies were dead 2 to 3 days after the loss of climbing ability. Experiments were performed at 29°C and similar results were obtained at 25°C (data not shown).

It is well known that mutations in genes that are acutely required in neuronal signaling show a paralytic phenotype at 37°C (Palladino et al., 2002). *Rbp9* mutants were tested for this phenotype. They were more sensitive to heat stress than wild-type flies (Fig. 2).

Dramatic change in neuronal cell integrity is not detected in *Rbp9* mutants

As *Rbp9* is expressed in post-mitotic adult neurons, the absence of *Rbp9* may cause overproliferation of neuronal cells or defects in neuronal differentiation. Since *elav* and *repo* are representative markers for neurons and glial cells, respectively (Halter et al., 1995; Robinow and White, 1988), we examined their expression levels in *Rbp9* mutants. Both the amount of the proteins and expression patterns did not change dramatically in mutant brains (data not shown).

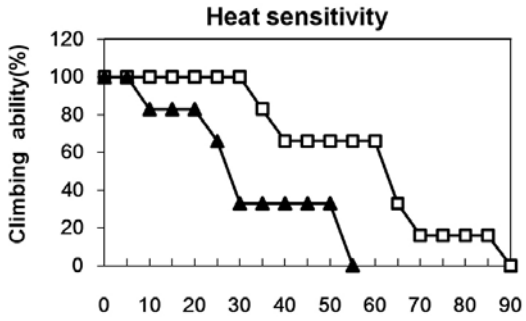

Fig. 2. A temperature-sensitive paralytic phenotype associated with *Rbp9* mutation. Wild-type (open squares) and *Rbp9* mutants (closed triangles) were tested for paralytic phenotype at 37°C. The locomotive activities of the flies at each time point after initiation of the heat treatment was measured.

Since model flies for human neurodegenerative diseases often show apoptosis of brain cells (Driscoll and Gerstbrein, 2003), we examined *Rbp9* mutant flies for neuronal cell death by TUNNEL assay. Some TUNNEL assay-positive cells were detected in both wild-type and mutant brains of 13-day-old flies. However, we could not detect any mutant brain-specific apoptotic cells (data not shown).

Genes whose expression levels change in *Rbp9* mutants

Gene expression profile analysis of *Rbp9* mutant flies were performed using microarrays containing 5,929 cDNAs. As whole adult flies contain many organs, only heads were dissected to specifically examine neuro-specific-gene expression. In addition, RNAs from male and female heads were prepared separately. Genes with changes in expression levels greater than 1.5-fold were selected from each experiment. Only genes that were selected in both females and males were considered final candidates. Three genes were identified as up-regulated genes and two genes including *Rbp9* were selected as down-regulated (Table 1). The DNA chip data were validated by either northern or RT-PCR analysis. All five genes identified from the microarray experiments were reconfirmed to be up- or down-regulated in the same pattern (Figs. 3A and 3B).

Except for *takeout* and *Rbp9*, the molecular or biological function of the other three genes identified from the microarray experiment has not been reported. Even when we considered the predicted functions of the uncharacterized genes based on protein domains, no apparent common function was found among the differentially expressed genes. As *takeout* was identified as a gene involved in feeding behavior (Sarov-Blat et al., 2000), we performed an experiment to test the ability to uptake food after starvation using food dye-containing food in wild-type and *Rbp9* mutant adult flies. We did not detect any noticeable defects in feeding behavior of *Rbp9* (data not shown). CG18316 has been identified as one of the genes differentially expressed in flies overexpressing polyglutamine (polyQ) proteins that undergo polyQ-mediated neurodegeneration (Nelson et al, 2005). However, its role has not been demonstrated, and we do not know how it affects phenotypes displayed in the *Rbp9* mutant. CG14521, which was down-regulated in *Rbp9* mutants, contain an immunoglobulin-like (Ig-like) domain. Ig-like domains are found in many proteins with diverse functions, including intercellular adhesions (Stanely et al., 2000).

Adhesion molecules are down-regulated in *Rbp9* mutants

When we dissected *Rbp9* mutant brains, we often noticed that the mutant brain looked somewhat less elastic than wild type. In addition, among the genes isolated, CG14521, which may function in cell adhesion, is down-regulated. Therefore, we examined genes that are known to be involved in intercellular adhesion. A dramatic decrease in the transcript levels of *Neurexin IV* and *Gliotactin* was noticeable. *Neuroglian*, *coracle*, and *Neurotactin* were also slightly decreased. On the contrary, *armadillo* transcript levels barely changed (Figs. 3C and 3D).

Table 1. Genes with altered expression in *Rbp9* mutants

	Gene ID	Biological processes	References
Up	*Takeout*	Behavioral response to starvation; circadian rhythm; feeding behavior; male courtship behavior;	Sarov-Blat et al. (2000) Stanewsky (2003)
	CG8889	Unknown function	
	CG18316	Unknown function	
Down	*Rbp9*	RNA binging protein germarium-derived egg chamber formation	Kim and Baker (1993) Kim-Ha et al. (1999)
	CG14521	Unknown function	

Blood-brain barrier is defective in *Rbp9* mutant brain

As *Neurexin IV* and *coracle* are critical components of tight junctions in mammalian and septate junctions in insects (Baumgartner et al., 1996), we examined whether the BBB is intact in *Rbp9* mutant heads by dye penetration analysis. Fluorescently labeled beads were injected into the abdomens of wild-type and mutant flies and at 18 and 40 h after injection, the amount of the beads incorporated into the brain was measured. In *Drosophila*, the BBB selectively transports molecules from the hemolymph; molecules with high molecular weights do not pass the barrier in wild-type flies. A dramatic increase in the penetration of beads was detected in *Rbp9* mutant brains (Fig. 4). Therefore, the BBB appears to be damaged in the *Rbp9* mutant brain.

Putative *Rbp9*-binding sites are found in *Neurexin IV* introns

As *Rbp9* contains RNA-binding motifs, its role has been predicted in the regulation of RNA processing. As *Rbp9* is found in the nuclei of neuronal cells, it may participate in the regulation of RNA splicing. We examined whether the consensus *Rbp9*-binding site we previously identified (Park et al., 1998), UUUXUUUU, is found in transcripts down-regulated in *Rbp9* mutants. Many putative *Rbp9*-binding sites were found in the introns of *Neurexin IV* (Fig. 5). *Rbp9* may participate in the splicing regulation of *Neurexin IV*.

DISCUSSION

Insulation from the surrounding environment is required for the proper function of the nervous system. The BBB provides this insulation in both vertebrates and invertebrates. A tight diffusion barrier and a complex array of transcellular transporters have been identified as components of the BBB (Mayer et al., 2009). In *Drosophila*, the BBB is composed of glial cells. As a tight diffusion barrier, pleated septate junctions formed by subperineural glial cells separate CNS from the hemolymph that circulates in the open circulatory system (Carlson et al., 2000). In

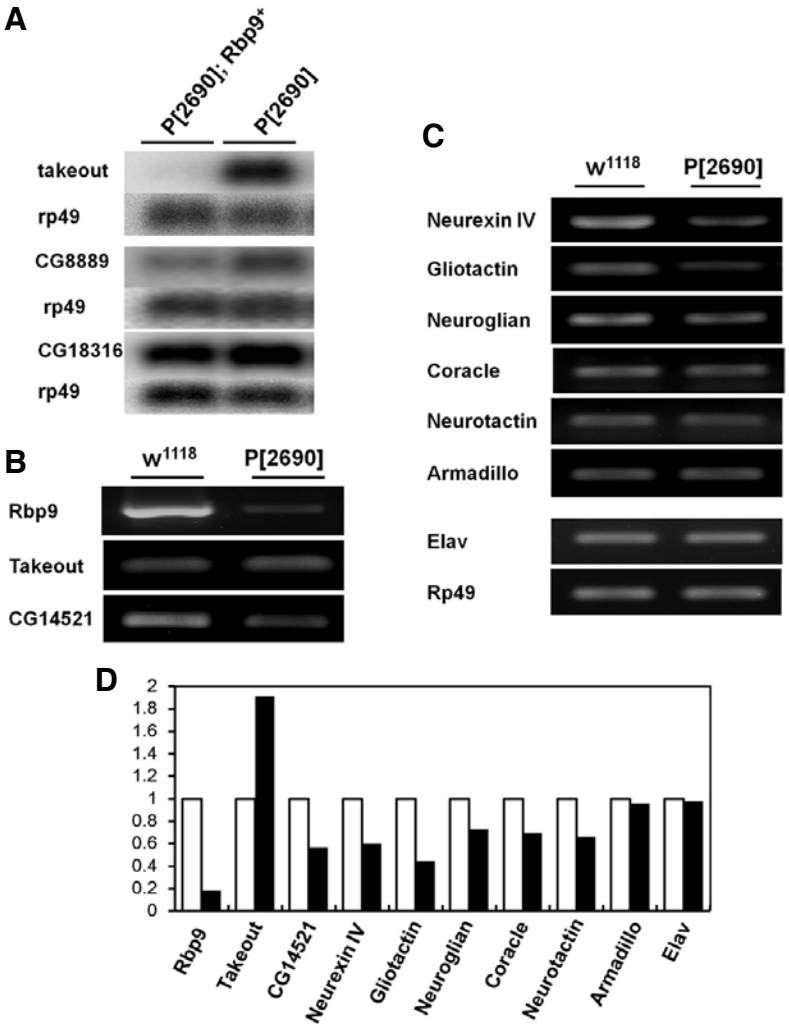

Fig. 3. Genes up or down regulated in *Rbp9* mutant heads. The DNA chip data was re-confirmed by either northern or RT-PCR analysis. (A) Genes selected as up-regulated in *Rbp9* mutant heads from the microarray analysis were re-confirmed by northern analysis. mRNA was prepared from adult fly heads, and *rp49* was used as an RNA loading control. Although the expression levels of *takeout* were consistently higher in *Rbp9* mutants, it showed some fluctuation (3- to 6.5-fold increase) by northern analysis. Data with the highest levels are presented. (B) Genes selected as down-regulated were tested by RT-PCR analysis. Total RNA was used. *Takeout* was tested again by RT-PCR analysis. (C) Gene expression analysis of various cell adhesion molecules in *Rbp9* mutant heads. A slight reduction in transcript level was observed in *Neuroglian*, *coracle*, and *Neurotactin*, while dramatic decreases were observed in *Neurexin IV* and *Gliotactin* transcripts. *Elav* which shares a high level of sequence similarity with *Rbp9*, did not show any decrease in transcript level. (D) Products in (C) were quantitated and the results are displayed graphically. The amount of each gene product from the mutant head (black bar) is shown as the relative ratio to that of wild type (white bar).

addition to this strong diffusion barrier, *Mdr65*, a fly ATP-binding cassette transporter, has been recently characterized to be required for chemical protection of the *Drosophila* brain (Mayer et al., 2009). As the BBB is formed during embryogenesis and begins to function at late embryonic stages, most studies on the formation and function of the BBB has been performed with the BBB formed during this period. Mutations in genes required for BBB formation have been shown to lead to paralysis of late stage embryos. Although the BBB is formed properly during early embryogenesis, it must be maintained throughout adult stages. *Moody* is one of the components required for proper BBB function. When *moody* gene expression is inhibited during adult stages, breakdown of BBB integrity occurs, resulting in defects in barrier function (Bainton et al., 2005). The integrity of the BBB appears to be formed reversibly as re-expression of the *moody* gene rescues the defective phenotype.

Although many possible target RNAs for *Rbp9* and its homologs have been identified, it is still unclear how *Rbp9* and its homologs affect neuronal function. Mutations in *Rbp9* do not result in embryonic lethality but a BBB-defective phenotype is detected right after eclosion. Although reversible destruction and reconstruction of BBB function has been observed using regulatory expression of the *moody* gene, the detailed process for this phenotype has not been analyzed. As dramatic morphogenetic changes occur during metamorphosis, modifications in BBB structure may occur during this period, and *Rbp9* might function in this process.

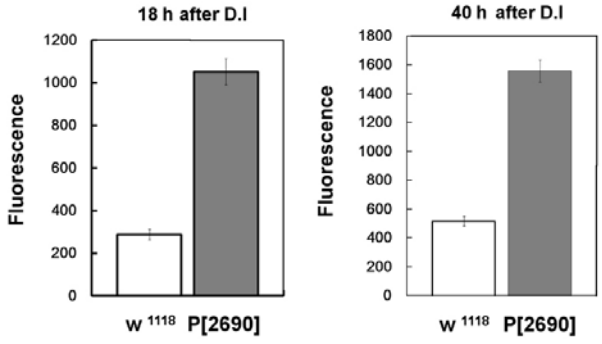

Fig. 4. Dye penetration assay to measure the integrity of the blood-brain barrier. Fluorescent dye was injected into the abdomens of 1-day-old flies. At 18 or 40 h after dye injection (D.I), brains were dissected from the flies and the level of the fluorescence was quantified. High levels of dye penetration into the brain were observed in *Rbp9* mutants. Therefore, the blood-brain barrier appears to be defective in *Rbp9* mutant brains. Similar results were obtained when we used 13-day-old flies (data not shown).

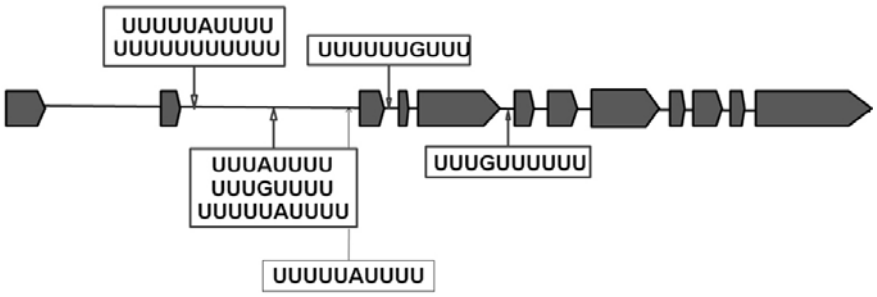

Fig. 5. Putative *Rbp9*-binding sites in *Neurexin IV*. Genomic organization of the *Neurexin IV* gene is schematically presented. One of the two alternatively spliced transcript isoforms (RA) is shown. Several copies of putative *Rbp9*-binding sites (UUUXUUUU) are observed in introns of *Neurexin IV* pre-mRNAs.

In *Rbp9* mutants, *Neurexin IV* expression is primarily affected, and putative *Rbp9*-binding sites are found in intron regions of *Neurexin IV*. In *Drosophila*, the BBB is composed of glial cells, and the role of *Neurexin IV* in BBB formation was only examined in glial cells. As *Rbp9* is expressed in neuronal cells and not glial cells, we are not sure how *Rbp9* participated in BBB formation. In other systems, there is some evidence that neuronal neurexins are involved in junction formation. Rat paranodin, a vertebrate homolog of *Neurexin IV*, is also implicated in barrier formation at the paranodal space of the node of Ranvier. In contrast to *Neurexin IV* expression in glial cells, paranodin is expressed in neurons (Einheber et al., 1997). In addition, neurexin III of elasmobranch fish is not only expressed in glial cells to form the BBB, but also localizes at the interface of axons and myelinating Schwann cells (Russell and Carlson, 1997). In vertebrates, endothelial cells of the brain capillaries are involved in BBB formation. Glial-cell-derived-neurotrophic factor secreted from astrocytes has been suggested to enhance the barrier function of tight junctions of the BBB formed by endothelial cells (Igarashi et al., 1999). The importance of neural lamella in barrier function has also been demonstrated (Stork et al., 2008). The role of neurons in barrier formation is not clear at this moment and more knowledge on the BBB formation process is required to understand how *Rbp9* participates in BBB formation.

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