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Secreted decoy of insulin receptor is required for blood-brain and blood-retina barrier integrity in *Drosophila*

*Jihyun Kim, Nuri Choi & Jeongsil Kim-Ha**
Department of Integrative Bioscience and Biotechnology, College of Life Sciences, Sejong University, Seoul 05006, Korea

Glial cells play important roles during neurogenesis and in maintaining complex functions of the nervous system. Here, we report the characterization of a gene, *Sdr*, which contains a putative insulin-like growth factor receptor domain and is required to maintain critical nervous system functions in *Drosophila*. *Sdr* is expressed in glial cells during embryonic and larval stages of development, but its role in adult flies is poorly understood. As insulin signaling is important throughout the lifespan in human, we investigated the *Sdr*'s role in adult flies. Our results demonstrate that *Sdr* is expressed on surface glial cells that surround the nervous system. Mutation of *Sdr* did not affect development but caused defects in locomotion and lifespan. *Sdr* mutants also showed increasingly severe defects in the blood-brain- and blood-retina-barriers as they aged. Therefore, we suggest a novel role of *Sdr* in maintaining the integrity of the blood-brain- and blood-retina-barriers in adult flies. [BMB Reports 2023; 56(4): 240-245]

INTRODUCTION

Insulin, insulin-like growth factor and target of rapamycin (TOR) signaling pathways are critical for proper regulation of systemic organismal growth under variable nutritional and environmental conditions (1). In mammals, insulin receptor (INSR) and insulin-like growth factor receptors (IGF-1R and IGF-2R) are present ubiquitously pre- and postnatally. The *Drosophila* genome encodes only one insulin receptor, called *Insulin-like receptor* (*InR*), which is initially translated into a single polypeptide and subsequently processed to generate the alpha and beta subunits of InR. The structure of *Drosophila CG3837*, *Secreted decoy of InR* (*Sdr*), is distinct from other insulin receptor genes in that it only encodes an alpha subunit. Evidence of the presence of truncated insulin receptor is accumulating in many insect species and evolutionary conservation has been reported (2). Because some of these truncated genes lack the transmembrane and/or tyrosine kinase domains, they could act as decoy receptors. In mammalian species, secreted variant isoforms of receptor tyrosine kinases including truncated variants of the insulin receptor, can be formed by alternative splicing (3).

Insulin/Insulin-like growth factor signaling pathways have primarily been investigated in liver, fat and skeletal muscles as they were reported to be direct target tissues of the signal. Recently the role of insulin signaling in central nervous system (CNS) to integrate nutrients, metabolites and hormonal signal, and to maintain glucose and lipid homeostasis and neurogenesis is emerging (4-7). Nervous system development requires proper organization, specification and interaction between neural and non-neural cells. The blood-brain barrier (BBB) arbitrates communication between the brain and the body. The Drosophila and vertebrate species BBBs are similar in many structural and functional aspects (8, 9). CNS physiology is sensitive to energy balance and it is reported that IGF-1 plays a neuroprotective role (10, 11). Despite its significance, little is known about the mechanism that regulates traffic of growth factors across the BBB. In this paper, we show that *Sdr* functions in glia, which constitute BBB and BRB, and plays a critical, novel role in maintaining the integrity of these barriers in adult flies.

RESULTS

Lifespan and locomotor activity are reduced in *Sdr* mutants

Drosophila Sdr is 42-49% homologous to *Drosophila InR*, human insulin receptor (hIR), and human insulin-like growth factor 1 receptor (hIGF-1R). However, the homology is restricted to the N-terminal ectodomains and *Drosophila Sdr* lacks the transmembrane and cytoplasmic tyrosine kinase domains found in other members of the IR/InR protein family. Role of *Sdr* in development or growth had been investigated mostly in embryonic and larval stages, and *Sdr* is reported to act as an insulin receptor decoy. To further investigate the function of *Sdr*, we generated an *Sdr* null allele with a 546 bp deletion by stimulating imprecise excision of the P-element located in the 1st exon of *Sdr* (location 14918660 according to Refseq NT_033777). The resulting allele, $Sdr^{\Delta 190}$, lacks 217 nucleotides

*Corresponding author. Tel: +82-2-3408-3644; Fax: +82-2-3408-4334; E-mail: jsha@sejong.ac.kr

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(nt) in the *Sdr* 5′UTR and 329 nt from the *Sdr* coding region, including the translation initiation site (Fig. 1A, B). Two mRNA transcripts were reported to be synthesized from the *Sdr* region, *Sdr*-RA and RB, that differ only in the 3′UTR and are predicted to generate the same polypeptide. However, because $Sdr^{\Delta 190}$ lacks a translation initiation site, flies carrying this allele are expected to lack SDR protein; whether a truncated Sdr transcript would be made was not clear. In fact, RT-PCR analysis using primers that hybridize to sequences in the 4th and 6th exons detected no mRNA from $Sdr^{\Delta 190}$ in adult flies (Fig. 2A, B). In addition, SDR protein was not detected by immunostaining $Sdr^{\Delta 190}$ adult tissues (Fig. 2F). These data confirm that $Sdr^{\Delta 190}$ is a null allele that generates no functional transcript or protein product.

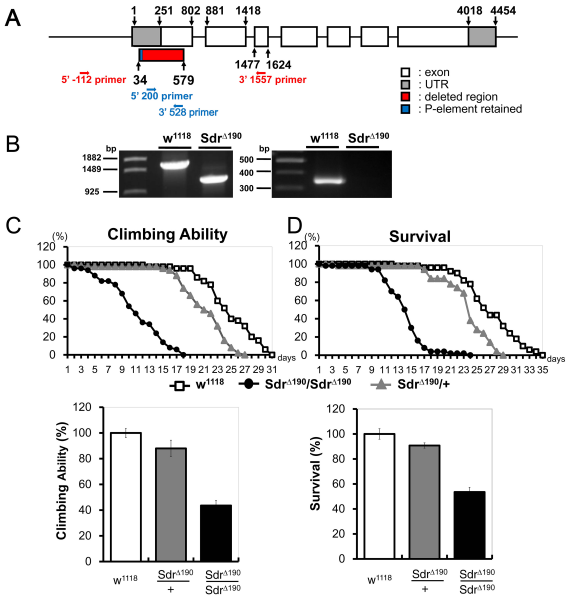

Fig. 1. Construction of *Sdr* mutant and defects in climbing and survival. (A) A schematic representation of the *Sdr* gene region in parental (GE22018) and $Sdr^{\Delta 190}$ genomes. The primers used to confirm the endpoints of the deletion caused by imprecise P-element excision are indicated and named by the coordinate of the 5′-end of the primer. The transcription start site is designated +1. (B) Genomic DNA from wild-type w^{1118} and $Sdr^{\Delta 190}$ mutant flies was PCR amplified using the two primer sets shown in (A). PCR amplification with the −112 and 1557 primers located outside of the deleted region produced a smaller product using $Sdr^{\Delta 190}$ genomic DNA (1123 bp) than using w^{1118} genomic DNA (1669 bp) as PCR template. PCR amplification with the 200 and 528 primers located within the deleted region only produced a product with only w^{1118} wild-type genomic DNA as template. (C, D) Climbing and survival were evaluated every other day throughout the fly's lifespan. Homozygous $Sdr^{\Delta 190}$ flies lost 50% of maximal climbing ability by day 10 and heterozygote $Sdr^{\Delta 190}$ flies lost 50% of maximal climbing ability by day 21. Survival and climbing ability data are presented as percent of initial (maximal) values. The bar graphs compare the time to 50% maximal capacity for survival or climbing ability for the indicated fly genotype relative to w^{1118} wild-type control.

$Sdr^{\Delta 190}$ flies did not show any obvious morphological defects during developmental stages from egg to adult. However, the lifespan of $Sdr^{\Delta 190}$ flies was shorter and locomotor activity was impaired relative to wild-type control flies. Although the ability of wild type flies to climb gradually declines with age, $Sdr^{\Delta 190}$ homozygous mutant flies lost the ability to climb at a much faster rate (e.g. time to 50% maximal climbing ability was 10 days or 24 days for $Sdr^{\Delta 190}$ or wild type, respectively) (Fig. 1C). The midpoint of the survival curve was 14 or 27 days for $Sdr^{\Delta 190}$ homozygous and wild type flies respectively, indicating that the lifespan of *Sdr* null flies was approximately 50% shorter than the lifespan of wild-type flies (Fig. 1D). $Sdr^{\Delta 190}$ heterozygous flies had similar defects in climbing ability and lifespan, but the effects were milder than in homozygous flies.

Sdr is expressed in the surface glia of the adult brain and eyes

We made polyclonal antibody against SDR protein. Because *Sdr* is homologous to *InR*, we tested whether the anti-SDR antibody cross-reacts with InR protein. Immunohistochemical studies were performed on IRP-Gal4>UAS-*Sdr* and IRP-Gal4>UAS-*InR*

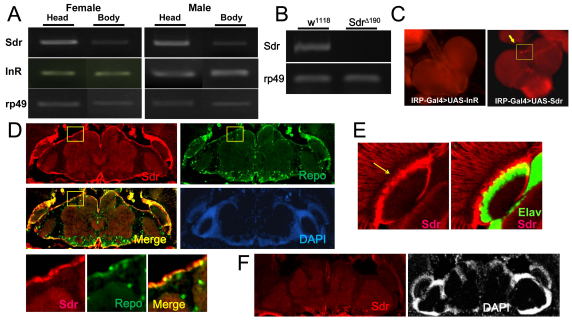

Fig. 2. Expression of the *Sdr* transcript and protein in wild-type and mutant adult tissues. (A) Adult male and female fly heads and bodies were separated. RNA was extracted from fly heads and bodies and used to estimate the expression of *Sdr*, *InR*, and *rp49* transcripts in the heads and bodies of male and female flies by RT-PCR. *Sdr* was primarily expressed in the fly head, while *InR* was expressed in the fly head and body. (B) *Sdr* RNA was quantified in whole-body extracts of w^{1118} and $Sdr^{\Delta 190}$ flies. (C) Specificity of rat anti-SDR antibody in larvae overexpressing SDR in neural cells. Larvae expressing IRP-Gal4>UAS-*Sdr* or IRP-Gal4>UAS-*InR* (control) were analyzed for immunoreactivity using rat anti-SDR antibody. The insulin-secreting cells in the pars intercerebralis are indicated with a box and arrow. (D) Localization of SDR protein in adult fly heads. Adult wild-type fly brains were prepared, sectioned horizontally, and stained with rat anti-SDR (red) or anti-REPO (green) antibody. Nuclei appear blue. Images showing SDR and REPO immunoreactivity were merged. Yellow signal in the cortical glial region indicates co-localization of red and green fluorescence. Boxed region in the upper images is shown at a higher magnification below. (E) Images show high immunoreactivity to anti-SDR antibody in the laminal cortex region (arrows), which does not colocalize with immunoreactivity to anti-ELAV (green), a neuronal marker. (F) Same as in (D) except flies expressing the $Sdr^{\Delta 190}$ null allele were immunostained with rat anti-SDR antibody (red). Nuclei are stained with DAPI (white).

larvae, in which SDR and InR are overexpressed in larval neuroendocrine cells, respectively. Anti-SDR antibody detected ectopic expression of SDR in IRP-Gal4 > UAS-*Sdr* larval brain but did not cross-react with overexpressed InR in IRP-Gal4 > UAS-*InR*. This confirms the specificity of the anti-SDR antibody we generated (Fig. 2C). In wild type adult flies, SDR immunoreactivity was detected in the cortex area which covers central nervous system (*i.e.*, central brain and medulla) and the laminal cortex of the eye. Co-staining with REPO or ELAV which are glial or neuronal markers, respectively, demonstrated glial-specific expression of SDR in wild type (Fig. 2D, E). On the contrary, SDR expression was no longer detected in $Sdr^{\Delta 190}$ flies (Fig. 2F).

Blood-brain-barrier and blood-retina-barrier defects in *Sdr* mutant

Because SDR is expressed in the glial cells in the CNS and the eyes of adult flies, we used $Sdr^{\Delta 190}$ mutant to investigate whether SDR is required for BBB and/or BRB function. The permeability of the BBB and BRB was examined by injecting tetramethylrhodamine-labeled dextran conjugates into the abdomen of adult flies. In healthy wild type flies, the labeled dextran conjugates do not cross the BBB or the BRB. We examined barrier leakage at the time when flies did not show any lethality but showed 50% decrease in climbing ability, the day 10 at 29°C. Significantly more dye penetrated the eyes of 10-day-old $Sdr^{\Delta 190}$ flies than wild-type flies (Fig. 3A). This phenotype was also observed at day 10 of $Sdr^{\Delta 190}$ flies raised at 25°C when mutants had normal survival and normal ability to climb. To confirm whether this leakage defect existed from the time when flies were born, we examined 2-day old young mutant flies. Most of the 2-day old young mutant flies did not show leakage of the dye into the eyes and only 30-40% of the flies showed small amount of leakage but at a greatly reduced level: indicating higher integrity of the BRB in 2-day-old $Sdr^{\Delta 190}$ flies. These results suggest that BRB integrity and function decrease with age in $Sdr^{\Delta 190}$ flies. This is consistent with the fact that the eyes of $Sdr^{\Delta 190}$ flies had abnormal patterns of rhodopsin staining (Fig. 3B). Rhabdomeres were significantly shortened and more disorganized than in control flies, and the severity of the defect increased with age.

To investigate the permeability and the integrity of the BBB in $Sdr^{\Delta 190}$ flies, tetramethylrhodamine-labeled dextran conjugates were injected into the abdomen of adult flies, brains were dissected, and the amount of dye that had crossed the BBB was measured with a spectrofluorometer. The results show approximately 10-fold higher fluorescence in $Sdr^{\Delta 190}$ brains than in wild type brains (Fig. 3C). These results indicate that the

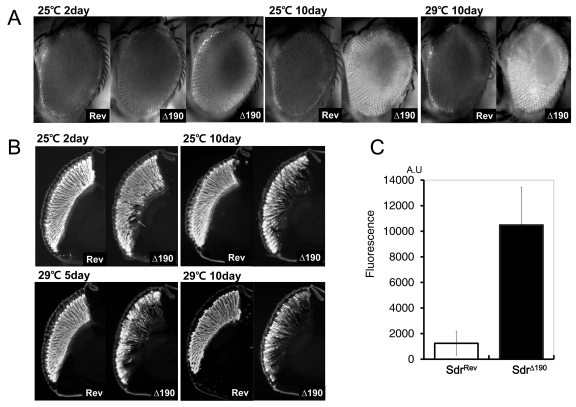

Fig. 3. Mutation in *Sdr* is associated with defects in BBB and BRB. (A) Tetramethylrhodamine-labeled dextran conjugate was injected into the abdomen of $Sdr^{\Delta 190}$ or $Sdr\Delta^{193}$ (Rev) control flies and then visualized or quantified as described in Materials and Methods. The images in (A) show the presence of leaked dye as a white signal. The temperature at which flies were maintained and the age of the flies in days at the time of analysis are indicated above each image. (B) The heads of $Sdr^{\Delta 190}$ or $Sdr\Delta^{193}$ (Rev) control flies were sectioned and rhabdomeres were stained with anti-rhodopsin antibody (white). The temperature at which flies were maintained and the age of the flies in days at the time of sectioning are indicated above each picture. (C) Fluorescence was quantified in heads of 10-day-old $Sdr^{\Delta 190}$ or $Sdr\Delta^{193}$ (Rev) control flies raised at 29°C using a spectrofluorometer. The results are presented in a bar graph using an arbitrary unit of fluorescence (labeled as A.U) on the Y-axis.

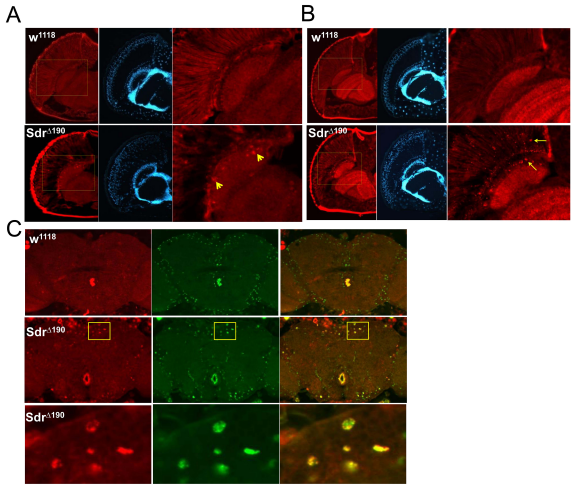

Fig. 4. Apoptotic markers are induced in *Sdr* mutant. Brain sections were prepared from 10-day-old wild-type (w^{1118}) and $Sdr^{\Delta 190}$ flies raised at 29°C and immunostained with antibodies to DRICE (A) or phosphorylated JNK (B). Positive immunoreactivity, visualized as red fluorescence, was detected in the laminal cortex of $Sdr^{\Delta 190}$ mutant flies but not in wild-type control. Nuclei appear blue (middle). (C) Same as in (A, B), except sections were immunostained with antibody to CASPASE3 (red) or REPO (green). Boxed region is enlarged in images to the right (A, B) or below (C). Arrows in the right images in (A, B) indicate strong positive signals. Lower right images in (C) show a yellow signal indicating co-localization of immunoreactivity to REPO (green) and CASPASE3 (red). The nuclei of cells with strong immunoreactivity to CASPASE3 appear to be swollen.

integrity of the BBB and BRB is significantly compromised in $Sdr^{\Delta 190}$ flies, leaving the brain and eye of these flies susceptible to damage and penetration.

Apoptosis is induced in aged *Sdr* mutants

To determine whether apoptosis is in progress in $Sdr^{\Delta 190}$ mutant, we examined expression and activation of markers of apoptosis including Decay (caspase3), Drice and p-JNK. Decay, Drice, and p-JNK were significantly induced in older mutant flies but not in wild type flies. Expression of Drice and p-JNK was higher in the laminal cortex of older $Sdr^{\Delta 190}$ flies (Fig. 4A, B) but caspase3 was not detected (data not shown). In contrast, caspase3 but neither Drice nor p-JNK was detected in the central brain cortex of $Sdr^{\Delta 190}$ flies (Fig. 4C). In addition, the nuclei of the caspase3-positive repo-positive $Sdr^{\Delta 190}$ glial cells appeared to be larger than the nuclei of repo-positive control glial cells, suggesting that nuclear swelling occurred in those caspase-positive repo-positive $Sdr^{\Delta 190}$ cells.

DISCUSSION

The human genome encodes 58 RTKs belonging to 20 RTK protein families. The *Drosophila* genome encodes 20 RTKs belonging to 11 of the 20 RTK protein families found in human (12). Mutations in genes encoding RTKs cause developmental disorders and cancer by altering various cell signaling and biological responses. As the availability of the complete genome sequences of various species has increased, the number of insect taxa with more than two *InR* copies are growing over the past decade (2). Among these *InR* paralogs, some encode truncated proteins that lack the tyrosine kinase domain. Both with and without transmembrane domain forms are also found. As they are expressed tissue- and sex-specifically (2), they would have distinct function but their function is mostly elusive and remain poorly characterized. In other animals including human, presence and expression of truncated RTK even in normal tissues has been reported (13, 14).

Recently, numerous effects of insulin signaling in brain are emerging. They include regulation of appetite, body temperature, reproductive function, systemic glucose metabolism (15, 16) and learning and memory (17, 18). Decreased expression of *InR* has been reported in the brains of animal models with Alzheimer's disease or Parkinson's disease, suggesting that defective or decreased insulin signaling could play a role in neurodegenerative disease (7).

In fact, we isolated *Sdr* mutant when we were searching for genes involved in neurodegeneration in adult flies (data not shown). Neuroprotective role and importance in brain homeostasis of IGF-1 has been reported (11, 19) and IGF can modulate blood vessel growth (20). To perform such functions, serum IGF-1 should cross the BBB in response to electrical, sensory, or behavioral stimuli (21). In general, the concentration of serum IGF-1 is stable; however, when serum IGF-1 increases, its concentration in cerebrospinal fluid is also reported to increase. This suggests the existence of a mechanism that regulates the transport of serum IGF-1 across the BBB.

Although *Drosophila Sdr* was described previously as a decoy of *InR* that regulates growth in fly larvae (22), it could also function in other processes and the amount of knowledge for its function is limited. When we examined dissociated primary cultures of embryonic cells, SDR was expressed in a punctate pattern in the cytoplasm of primary cultures of *Drosophila* embryonic cells (data not shown). Thus, translated SDR seem to exist in vesicular form until they are secreted. Trafficking of SDR-containing vesicles could be regulated to maintain homeostasis of the insulin signaling in the brain. The *Drosophila* genome encodes 8 *insulin-like peptides* (*ilps*). Seven of these peptides, ILP1 to ILP7, are reported to bind InR protein and repress growth inhibition and stress response pathways (23). In contrast, ILP8 binds to relaxin receptor homolog LGR3 to slow growth in response to injury (24, 25). Co-immunoprecipitation studies show that *Drosophila* SDR interacts with all *Drosophila* ILPs except ILP4 and ILP8 (22).

Brain activity depends on access to circulating oxygen and nutrients. Endothelial transport of oxygen and glucose and brain activity is facilitated by a neurovascular mechanism (26). Insulin signaling in the adult brain is required as insulin inhibits neuronal apoptosis and tau phosphorylation (19). SDR may continuously bind to and dissociate from ILPs (*i.e.*, like IGFBP), releasing them when they are needed. Alternatively, secretion of SDR might be regulated, so that it is secreted from glial cells when it is needed. As high level of SDR is constantly synthesized in the brain, a high concentration of SDR may be required to rapidly bind to ILPs and block their function. And these processes may be required to maintain BBB and BRB in adult flies.

MATERIALS AND METHODS

Longevity and climbing tests

Flies were collected as newborn adults over a 12-hour time window. Ten newborn flies were placed in a fresh vial and stored for 1 day at 25°C before being used in climbing and survival tests. During the experiment, flies were maintained at 29°C. After negative geotactic pressure was applied, the number of flies that climbed to the top of the vial within 20 seconds was counted and recorded.

Production and purification of rat anti-SDR polyclonal antibody

To construct an SDR overexpression plasmid, a 730 nucleotide DNA fragment was amplified by PCR using LD44769 (the EST clone of the SDR 5′ region) as template DNA and Pfu polymerase (Agilent/Strategene) and the following two primers: 5′ CGGGATCCGTGGCAGATGTATC3′ and 5′GGAATTCTGCGA TAGTCGTGATTG3′. *BamH*I and *Eco*RI digested PCR product was then cloned into the pRSET A vector. The resulting plasmid was used to express and purify 39 kDa recombinant SDR

protein, which was used for primary and booster injections into rats to raise rat anti-SDR polyclonal antibodies. Recombinant SDR was coupled to CNBr-activated sepharose 4B (Amersham Biosciences, NJ, USA), and serum taken from rats after the booster injection was applied to this column. Bound antibodies were eluted to recover affinity-purified rat anti-SDR antibodies. Affinity-purified antibody was tested for specificity and cross-reactivity with *Drosophila* InR protein, as follows. InR and SDR were overexpressed in neuroendocrine cells of fly larvae using the IRP-Gal4 system (IRP-Gal4 > UAS-*InR* or IRP-Gal4 > UAS-*Sdr*). Ectopic expression of SDR was detected in SDR-overexpressing larvae but no immunoreactivity was detected in InR-overexpressing larvae (*i.e.*, IRP-Gal4 > UAS-*InR*) (Fig. 2). Therefore, the rat anti-SDR antibody is specific for *Drosophila* SDR and does not cross-react with InR in *Drosophila* larvae.

Construction of Sdr null mutant and revertant

GE22018 flies were purchased from Genexel (Taejun, Korea). In the genome of GE22018 flies, a P-element is inserted in the 1st exon of SDR at location 14918660 (RefSeq NT_033777). To mobilize the P-element in the GE22018 genome, GE22018 flies were crossed to flies harboring P[Δ2-3] transposase, and progeny of this cross were monitored for loss of eye pigmentation as a marker of P-element excision. To determine whether the P-element was excised precisely or imprecisely, we bred homozygous flies with putative P-element excisions, isolated their genomic DNA, and PCR amplified and analyzed the remaining *Sdr* gene sequences in each fly strain. The upstream primer used for this analysis started at −112 (relative to the transcriptional start site +1) and the downstream primer started at nt 1557 downstream of the transcriptional start site of *Sdr*. The DNA sequence of the products of PCR amplification was determined and the P-element excision sites were identified. The DNA sequence of one fly strain revealed imprecise P-element excision. This fly strain, $Sdr^{\Delta 190}$, had a deletion from nt 14918661 to nt 14919173 of the genomic DNA, according to the Refseq locus AE014297, and it retained 14 nt of P-element DNA (*i.e.*, CATGATGAAATAAC). The DNA sequence of a second fly strain revealed precise excision of the P-element. This fly strain, $Sdr^{\Delta 193}$ was considered to be a phenotypically normal revertant of the parental GE22018 strain, and where indicated, the revertant strain (Rev) was used as a wild-type control.

Cryosectioning and Immunostaining fly brain

The heads of adult flies were fixed in 4% paraformaldehyde for 10 min, transferred to embedding media, and then cryosectioned at a thickness of 15 μm. Sections were placed on lysine-coated slides (Superior Marienfield, Lauda-Konigshfen, Germany) and immunostained as described in Kim *et al*. (27). Briefly, slides were fixed in 4% paraformaldehyde for 10 min at room temperature (RT), washed 3× for 5 min with 50 mM Tris-Cl pH7.5, 15 mM NaCl, 0.1% BSA, 0.1% Triton X-100 (1× TNBT), incubated with 5% goat serum for 30 min and then incubated with primary antibody overnight at 4oC. Finally, slides were washed with 1× TNBT for 30 min, and then incubated with secondary antibody for 2 h at RT. Slides were mounted with Vectashield and visualized under a Zeiss Microscope (Axioplan2, Carl-Zeiss, Oberkochen).

Permeability tests of the BBB and BRB

A solution containing tetramethylrhodamine-labeled dextran conjugates (10,000 MW, Molecular Probes) was loaded into a glass capillary prepared with a needle puller (PN-30, Narishige Scientific Instrument Lab, Tokyo), and injected into the abdomen of adult flies (150 nl/fly). After 2 h, injected flies were dissected, and the brains of 2 flies were placed into a single well containing 50 μl 0.1% SDS. The fluorescence from each well was measured using a SpectraMax Gemini EM microplate spectrofluorometer (Excitation = 555 nm, Emission = 580 nm). Each experiment was repeated at least 8 times. The amount of fluorescent dye in eyes of injected flies was estimated by examining eyes under a Zeiss Microscope (Axioplan2, Carl-Zeiss, Oberkochen). Experiments were repeated at least 10 times for each experimental group.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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