Oatp58Dc contributes to blood-brain barrier function by excluding organic anions from the Drosophila brain

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Seabrooke S, O’Donnell MJ. Oatp58Dc contributes to blood-brain barrier function by excluding organic anions from the Drosophila brain. Am J Physiol Cell Physiol 305: C558–C567, 2013. First published June 26, 2013; doi:10.1152/ajpcell.00408.2012.— The blood-brain barrier (BBB) physiologically isolates the brain from the blood and, thus, plays a vital role in brain homeostasis. Ion transporters play a critical role in this process by effectively regulating access of chemicals to the brain. Organic anion-transporting polypeptides (Oatps) transport a wide range of amphipathic substrates and are involved in efflux of chemicals across the vertebrate BBB. The anatomic complexity of the vascularized vertebrate BBB, however, creates challenges for experimental analysis of these processes. The less complex structure of the Drosophila BBB facilitates measurement of solute transport. Here we investigate a physiological function for Oatp58Dc in transporting small organic anions across the BBB. We used genetic manipulation, immunocytochemistry, and molecular techniques to supplement a whole animal approach to study the BBB. For this whole animal approach, the traceable small organic anion fluorescein was injected into the hemolymph. This research shows that Oatp58Dc is involved in maintaining a chemical barrier against fluorescein permeation into the brain. Oatp58Dc expression was found in the perineurial and subperineurial glia, as well as in postmitotic neurons. We specifically targeted knockdown of Oatp58Dc expression in the perineurial and subperineurial glia to reveal that Oatp58Dc expression in the perineurial glia is necessary to maintain the barrier against fluorescein influx into the brain. Our results show that Oatp58Dc contributes to maintenance of a functional barrier against fluorescein influx past the BBB into the brain.

organic anion-transporting polypeptide; Drosophila; subperineurial; perineurial; fluorescein

ORGANIC ANION-TRANSPORTING polypeptides (Oatps), members of the SLC21 family, transport a wide range of amphipathic substrates and are involved in uptake of substrates into cells. In humans, Oatp transporters are expressed in multiple tissues, including liver, kidney, lung, heart, intestine, placenta, testis, and brain (55). In mouse, four solute carrier organic anion transporters (Oats), including one Oat (SLC22A8) and three Oatps (SLCO1a4, SLCO1c1, and SLCO2b1), have been shown to be transported by Oatps transfected into Chinese hamster ovary cells (14, 22) and by Drosophila Malpighian tubules, as tight junctions between capillary endothelial cells that lack fenestrations and have reduced pinocytosis, thus contributing to maintenance of a homeostatic environment for brain cells (1, 30, 45, 49). The permeation of compounds across the barrier is further regulated by ion transporters, which regulate transcellular movement of compounds (2, 20, 21, 52, 58). The effectiveness of the BBB at regulating access of chemicals to the brain has provided many challenges for the development of drugs targeting the central nervous system (CNS) (2, 20, 52). Indeed, there is increasing evidence that ion transporters, such as the Oats, play a fundamental role in limiting access of drugs to the brain (20, 35, 36, 52, 54). Overcoming challenges of CNS drug development requires integrative research from numerous disciplines, as well as improved in vivo models of the BBB (42).

In Drosophila, the brain is separated from the open circulatory system by glial epithelial cells known as surface-associated glia. The perineurial and subperineurial glia constitute the surface-associated glia, which entirely ensheath the CNS and make up the BBB. This CNS-humoral interface in Drosophila shares many conserved properties with the vertebrate BBB (15). The subperineurial glial layer is believed to be the principal diffusion barrier (51), positioning this layer as essential in chemoprotection. More recently, evidence has shown that expression of the Drosophila ATP-binding cassette transporter Mdr65 in the subperineurial glia is important for chemoprotection (37). In addition to the surface-associated glia, cortex- and neuropil-associated glia are found within the adult CNS (50).

This less complex BBB allows the use of fluorometric assays to rapidly characterize the kinetics of transport across the invertebrate BBB. Methods have been developed to track compounds injected into the body of a live fly and measure the amount of the compound in the brain. Such studies combine molecular, genetic, and physiological tools to study ion transport physiology and paracellular permeability at the Drosophila BBB in vivo (6, 37, 44, 47, 51).

Eight putative Oatp transporters are found in the Drosophila genome and are named according to their location on the chromosome. Microarray data available on FlyAtlas (http://flyatlas.org/) indicate that two of the eight Oatp transporters, Oatp58Dc and Oatp74D, are enriched in the adult brain. Oatp58Dc expression has been shown to be altered in the Malpighian tubules in response to dietary loading with the small organic anion salicylate (11). Oatp58Dc is also involved in fluorescein transport by the Malpighian tubules (12).

This research was undertaken to further our understanding of the barrier properties of the BBB, with Drosophila used as a model. We have used fluorescein, a small organic anion known to be transported by Oatps transfected into Chinese hamster ovary cells (14, 22) and by Drosophila Malpighian tubules, as...
a probe for quantitative analysis of organic anion transport. We used genetic and pharmacological manipulation, along with confocal analysis and an in vivo microinjection assay similar to the technique developed by Bainton et al. (6), to identify Oatp58Dc as an ion transporter involved in the efflux of small organic anions from the Drosophila brain.

MATERIALS AND METHODS

Drosophila stocks. All genetic crosses were made on standard yeast medium and kept at 22°C. Tissue-specific gene expression was achieved using the Gal4/UPsream activating sequence (UAS) expression system (8). UAS-oatp58DcΔ946-9. RNAi, [oatpRNAiΔ9469], FlyBase Stock Center (FBst) record no. FBst0463041) is inserted on the third chromosome and UAS-oatp58DcΔ946-9RNAi, (oatpRNAiΔ9469), FBst0463042) is inserted on the second chromosome. These RNA interference (RNAi) constructs allow for tissue-specific knockdown of the Oatp58Dc, while UAS-moodyRNAi (moodyRNAi; FBst0452950) allows for tissue-specific knockdown of the G protein-coupled receptor Moody. These flies were obtained from the Vienna Drosophila RNAi Center (16). oatp58DcΔ946-9RNAi (oatpRNAiΔ9469), FBst0030642) is a loss-of-function allele. The g4 driver repoGal4/TM3, Sb (FBst0007415) drives expression in all glia except midline glia. elavGal4 drives expression in neurons (FBst0008760). UAS-CD8-GFP (FBst0005130) was used to express green fluorescent protein (GFP) in a tissue-specific manner. oregenR (oreR) and yw were used as controls. These flies were obtained from the Bloomington Drosophila Stock Center. NP629Gal4/Cyo (pgGal4) and NP2276Gal4/Cyo (spgGal4) drive expression in the adult perineurial and subperineurial glia, respectively (5), and were obtained from the Drosophila Genetic Resource Center.

Topology analysis. The predicted sequence of transmembrane domains was determined using the Ensembl Genome Browser (http://www.ensembl.org/index.html). Orientation of the cytoplasmic and extracellular sides of the protein was predicted using the TMHMM server (version 2.0, http://www.cbs.dtu.dk/services/TMHMM/). Putative sites of N-linked glycosylation were determined using the GPP server (25).

Quantitative RT-PCR. For each sample, RNA was isolated from five adult brains using the PureLink RNA MicroKit (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s protocol for purifying RNA from animal tissues. Brains were homogenized to release the fluorescein from the tissue into the solution and frozen at −20°C. The six dissected brains were pooled in 0.5 μl of 0.2% SDS in dH2O containing 0.5% methylene blue, which was added to eliminate the autofluorescence associated with the Drosophila retina (7). The corresponding six bodies were placed in a tube containing 100 μl of 0.2% SDS in dH2O. The tissues were homogenized to release the fluorescein from the tissue into the solution and frozen at −20°C. The six dissected brains were pooled in 0.5 μl of 0.2% SDS in dH2O containing 0.5% methylene blue, homogenized, and frozen. To confirm that there were no significant differences in the excretion of fluorescein from the bodies, three bodies including intact heads were pooled in 50 μl of 0.2% SDS in dH2O for each genotype dissected. Flies were pooled to bring the fluorescence content to an optimal range for measurement.

Confocal imaging of capillary tubes. All samples except dissected brains were thawed, and 1 μl of each sample was pipetted into depressions cut into a Sylgard-coated dish that had been filled with parafin oil. Samples were kept for 2 h, which allowed time for solid debris to float to the surface of the sample. The sample for confocal imaging was taken up in an optically flat rectangular glass capillary tube following methods described by Leader and O’Donnell (32). Briefly, a rectangular 0.05-mm thick 0.5-mm wide capillary tube (VitroTubes, Mountain Lakes, NJ) was touched to the sample via an approach from the side where the sample was clear of solid debris. The sample was drawn into the tube by capillary action and bracketed by parafin oil on either side. Capillary tubes containing samples and standards of known concentration were placed on glass microscope slides for viewing under the confocal microscope. Samples from tubes with dissected brains were thawed and directly drawn up into capillary tubes for imaging.

All images were collected on a Leica TCS SP5 confocal microscope. For imaging of capillary tubes, slides were placed under a HCX PL APO CS 10.0×0.40 dry UV objective. The argon laser was set to 40% of maximum, and the 488-nm laser line was set to 15% power. A triple dichroic (TD 488/543/633-nm) beam splitter was utilized, and the photomultiplier tube detectors were set to detect 500- to 535-nm wavelengths. Images were collected at 400 Hz, and the pinhole was set to 1 airy unit. The image of the brightest standard was optimized using the overlay/underlowlow settings. Settings were not changed between samples. A standard curve was made at the beginning of each confocal session. The image was focused to the middle of the capillary tube along the z-axis, corresponding to the maximal fluorescence intensity as described by Leader and O’Donnell (32), and a single 4.12-μm slice was collected.

Analysis of fluorescein content in capillary tubes. A ~400 × 1,000-μm rectangle was digitally added to the image of the rectangular capillary tube to calculate pixel intensity in ImageJ version 1.43u [National Institutes of Health (http://rsbweb.nih.gov/ij/)]. If debris was visible in the image, the freehand selections tool was used to measure the pixel intensity in a ~0.4-mm² area that was free of Rp49 primer sequences were used previously by Seabrooke and Stewart (48) as a reference gene in the Drosophila larval brain.

The following primer sequences were used: 5’-CTGGGATATTCCGGCCCAAGTG-3’ (forward) and 5’-CTATCGAAAAACCCACCCCGAAA-3’ (reverse) for oatp58Dc and 5’-CTCGGTTCGACGGAACCAAA-3’ (forward) and 5’-GACAACTCTCTTGCGCCTTCT-3’ (reverse) for Rp49.

Microinjection protocol. Glass capillaries (1.5 mm OD; A-M Systems, Carlsborg, WA) were pulled to tip diameters of ~3 μm on a Narishige vertical pipette puller and forward-filled with 0.2 μl of 300 μM fluorescein (catalog no. F-6377, Sigma-Aldrich) in Drosophila saline (mmol/l: 117.5 NaCl, 20 KCl, 2 CaCl2, 8.5 MgCl2, 10.2 NaHCO3, 4.3 NaH2PO4, 8.6 HEPES, 10-l-glutamine, and 20 glucose), which was titrated to pH 7 with NaOH.

The fluorescein-saline solution was injected into the hemolymph between the thorax and abdominal segments of adult flies that had been lightly anesthetized with CO2. The flies were left to recover for 15 min; then they were decapitated with a razor blade, or their brains were dissected following the dissection protocol outlined by Wu and Luo (59). Dissections were completed in <120 s. The six heads were pooled in 3 μl of 0.2% SDS in dH2O containing 5% methylene blue, which was added to eliminate the autofluorescence associated with the Drosophila retina (7). The corresponding six bodies were placed in a tube containing 100 μl of 0.2% SDS in dH2O. The tissues were homogenized to release the fluorescein from the tissue into the solution and frozen at −20°C. The six dissected brains were pooled in 0.5 μl of 0.2% SDS in dH2O containing 0.5% methylene blue, homogenized, and frozen. To confirm that there were no significant differences in the excretion of fluorescein from the bodies, three bodies including intact heads were pooled in 50 μl of 0.2% SDS in dH2O for each genotype dissected. Flies were pooled to bring the fluorescence content to an optimal range for measurement.

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debris. The pixel intensities of the standards were measured and fit to a line using linear regression. For the standard curves, $R^2$ was $\geq 0.98$. The standard curve was used to convert pixel intensity values from the samples to micromolar concentrations of fluorescein. The total volume in which the heads, brains, or bodies had been dissolved was used to convert the micromolar concentration of fluorescein to picomoles. The contents per head, per brain, and per body were calculated by dividing the micromolar concentration by the number of different animals used per tube (6 or 3). Background fluorescence images were captured using un.injected flies and subtracted from all other values.

**Immunocytochemistry.** Brains were dissected, fixed, and stained following the protocol described by Wu and Luo (59). Rabbit anti-Oatp53Dc [provided by Dr. Julian Dow (57)] was used at a dilution of 1:500. Cy3-labeled sheep anti-rabbit antibody (Sigma-Aldrich, Oakville, ON, Canada) was used at a dilution of 1:200. Chicken anti-GFP (Abcam, Toronto, ON, Canada) was used at a dilution of 1:1,000. Alexa Fluor 488 goat anti-chicken (Invitrogen Molecular Probes, Burlington, ON, Canada) was used at a dilution of 1:400.

Images were collected on a Leica TCS SP5 confocal microscope with a HCX PL APO CS 63.0 ×1.40 oil UV objective with zoom. Images were collected by sequential scanning using argon 488-nm and helium-neon 543-nm lasers. The argon laser was set to 40%, and 15% power was used. A TD488/543/633-nm filter was selected, and filters were set to collect wavelengths between 500 and 535 nm. The helium-neon 543-nm laser was set to 40% power. The TD488/543/633-nm filter was used, and wavelengths between 555 and 620 nm were collected. Images were optimized using the overglow/underglow settings. The pinhole was set to 1 airy unit, which collected a 6.23-μm-thick image. A 2,048 × 2,048-pixel image with 16-bit resolution was captured using a scan speed of 100 Hz and a line averaging of 2.

**Statistical analysis.** Statistical analysis was completed in GraphPad Prism 4.0. For comparison between two means, an unpaired two-tailed t-test was completed. For one-way comparison between multiple groups, one-way ANOVA was conducted. A Newman-Keuls multiple comparisons posttest was completed to identify significant differences. For qRT-PCR, a pair-wise fixed reallocation randomization test with 2,000 randomizations was completed using REST version 2 software. $P < 0.05$ was taken as significant. Values are means ± SE.

**RESULTS**

In previous studies that have developed the microinjection assay to study permeability of the BBB, movement of a compound into the brain was visualized by looking through the eye is limited to flies containing the repoGal4 construct (Fig. 2A). We also determined fluorescein concentration in the bodies (Fig. 2B) and found no significant difference between control flies and flies with reduced Moody transcript. These results confirm that changes in penetration of fluorescein into the brain could be detected by utilizing the whole head and calculating fluorescein concentration in the fly.

To determine whether ion transporters also contribute to the barrier against fluorescein, Drosophila oreR flies were injected

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Fig. 1. Fluorescein concentration in the samples. A: sample image of an optically flat capillary tube filled with SDS, methylene blue, and fluorescein released from homogenized tissue. Note small amount of tissue debris in upper and lower regions of the image. Pixel intensity of the image was measured using ImageJ. B: standard curve of known fluorescein concentrations showing a linear fit used to calculate fluorescein concentrations from images in A. A standard curve was imaged at the beginning of each confocal session, and $R^2 \geq 0.98$ was accepted. Overglow/underglow settings were adjusted using a capillary containing 3 μM fluorescein and were not changed for the duration of the session.

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with 300 μM fluorescein in saline or 300 μM fluorescein in saline + 3 mM salicylate, a nonfluorescent competitive inhibitor of organic anion transport. Flies were left for 15 min before the heads were removed, and the tissues were homogenized. In response to salicylate coinjection (Fig. 2C), fluorescein concentration in fly heads showed a significant increase relative to the flies injected with fluorescein alone, whereas no significant difference was found in the corresponding bodies (Fig. 2D). These results confirm that ion transporters are present in the Drosophila brain and are important for maintaining a barrier against fluorescein influx into the brain. We therefore investigated whether Oatp58Dc plays a role in fluorescein efflux from the Drosophila brain.

Topology of Oatp58Dc. Although Oatp58Dc is predicted to share the structural features of the Oatp family, the predicted topology of this transporter has not been reported. Oatp58Dc is a transmembrane protein with 12 predicted transmembrane domains. It contains the signature sequence D-X-RW-(I,V)-GAWW-X-G-(F,L)-L, which is common to the Oatp family, with two distinct differences: 1) in the Oatp58Dc signature sequence, the 5th amino acid is leucine, instead of the more commonly found isoleucine or valine, and 2) the 12th amino acid is tryptophan, instead of the more commonly found phenylalanine or leucine. The signature sequence spans extracellular loop 3 into transmembrane domain 6. Consistent with the Oatp family, extracellular loop 5 is large and cysteine-rich, containing 10 cysteine residues, similar to other Oatp sequences (23, 24, 26, 38, 41). Extracellular loops 2 and 5 contain putative N-linked glycosylation sites, consistent with the Oatp family (Fig. 3).

Localization of Oatp58Dc in the adult Drosophila brain. Although expression of Oatp58Dc in the adult brain has been confirmed by Western blotting (57), localization of this transporter in the brain has not been determined. We therefore used immunocytochemistry to determine localization of Oatp58Dc in the adult Drosophila brain. We first labeled the brain for Oatp58Dc and found that it was widely distributed on the surface of the brain. To identify which cell types express Oatp58Dc, we drove expression of the UAS-CD8-GFP insert

Fig. 2. Fluorescein concentration in Drosophila head ([Fluorescein]Head) and body ([Fluorescein]Body) 15 min following fluorescein injection. A: significant increase in fluorescein concentration in the head was observed when blood-brain barrier (BBB) permeability was increased using moodyRNAi/+;repoGal4/+ (moodyRNAi) to reduce Moody expression. B: no significant increase in fluorescein concentration in the body was observed when BBB permeability was increased using moodyRNAi to reduce Moody expression. C: competitive inhibition by coinjection of salicylate with fluorescein resulted in a significant increase in fluorescein concentration in the head. D: no significant differences were found in fluorescein content of the body when salicylate was coinjected with fluorescein. Values are means ± SE; n = 5 for each sample. *P < 0.05 (by unpaired, 2-tailed t-test).

Fig. 3. A: predicted topology of organic anion-transporting polypeptide (Oatp) 58Dc (Oatp58Dc). Extracellular and intracellular sides of Oatp58Dc are labeled. Horizontal lines indicate the plasma membrane, and transmembrane domains are color-coded according to the highlighting colors in B. Signature sequence of Oatp proteins is shown in red. Y indicates N-linked glycosylation sites, and orange filled circles are cysteine residues. B: sequence of Oatp58Dc. Highlighted sequences correspond to transmembrane domains highlighted in A. Red lettering indicates signature sequence of Oatp proteins; yellow lettering indicates predicted sites of N-linked glycosylation.
in specific cells found at the brain surface and double-labeled the brain for GFP and Oatp58Dc. UAS-CD8-GFP drives expression of a membrane-bound marker labeling the plasma membrane of cells. We used the tissue-specific drivers pgGal4, spgGal4, and elavGal4 to express the UAS-CD8-GFP insert in perineurial glia, subperineurial glia, and postmitotic neurons, respectively. Oatp58Dc expression was found in all three layers: perineurial glia, subperineurial glia, and postmitotic neurons (Fig. 4). To confirm the specificity of the Oatp58Dc antibody, we labeled control and oatp30642 mutant flies with the Oatp58Dc antibody and found an overall reduction of immunofluorescence labeling across the three immunoreactive cell types (Fig. 5). Thus Oatp58Dc is well positioned to efflux organic anions from the adult Drosophila brain.

Quantification of mRNA expression in transgenic and mutant alleles of oatp58Dc. qRT-PCR was used to confirm that the transgenic and mutant alleles that target oatp58Dc reduced expression of the mRNA transcript (Fig. 6). Within each genotype, expression levels were first normalized to the reference gene Rp49. All the genotypes were then compared with repoGal4/+ on a log-2 scale. Expression of oatp58Dc in yw flies was not significantly different from repoGal4/+. This indicated that expression of oatp58Dc remained consistent in control genotypes. We next determined the relative expression of oatp58Dc in adult brains of two transgenic RNAi constructs and one loss-of-function allele of oatp58Dc. repoGal4 was used to drive expression of oatpRNAi39469 (repo K/D 39469) and oatpRNAi39470 (repo K/D 39470) in glial cells. In both

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**Fig. 4.** Immunocytochemical analysis of Oatp58Dc localization in cell layers of the brain. **Left:** staining for green fluorescent protein (GFP, green). **Middle:** staining for Oatp58Dc (red). **Right:** merged image. **A:** perineurial glia were labeled using the UAS-CD8-GFP insert driven by the perineurial-specific driver pgGal4 (green). **B:** higher magnification of image in A showing Oatp58Dc staining in the perineurial glia. Arrow shows the edge of the perineurial glial layer. **C:** subperineurial glia labeled using the UAS-CD8-GFP insert driven by the subperineurial-specific driver spgGal4 (green). **D:** higher magnification of image in C showing colocalization of Oatp58Dc (red) with subperineurial glia (green). Note immunoreactivity for Oatp58Dc on the outside of the subperineurial glia, consistent with localization of Oatp58Dc in the perineurial glial layer. **E:** postmitotic neurons labeled using the UAS-CD8-GFP insert driven by elavGal4 (green) showing localization of Oatp58Dc in these neurons.
Fig. 5. Cellular localization of Oatp58Dc immunoreactivity in the brain. To confirm that immunofluorescence labeling in the brain could be attributed to Oatp58Dc, we looked for a reduction in overall immunoreactivity in the brain using oatp10642. Because OregonR (oreR) was shown to have the same physiology of fluorescein transport across the brain as repoGal4/+ (data not shown), it was used as control. Brains for control and oatp10642 lines were labeled simultaneously and visualized during the same confocal session at the same settings. An overall reduction of fluorescence intensity was observed in oatp10642 flies. A: immunoreactivity of the Oatp58Dc antibody in the brain of oreR flies. B: immunoreactivity of the Oatp58Dc antibody in the brain of oatp10642 loss-of-function flies. C: fluorescence intensity plotted across the brain starting from just outside the edge of the brain to the edge of the immunoreactive neurons observed using elavGal4 (Fig. 4E). Five profiles were taken for each image and averaged. Averages were collected from 6 individual brains for control and oatp10642 and used to generate the fluorescence intensity profile. A reduction in fluorescence intensity is observed throughout the area labeled by the Oatp58Dc antibody. Because oatp10642 does not result in a complete knockout of Oatp58Dc expression as determined by quantitative RT-PCR, we would not expect a complete loss of fluorescence labeling. The greatest decrease in fluorescence intensity is observed at the outer edge of the brain, suggesting that Oatp58Dc is more highly expressed in the BBB than in the neurons. However, the overall reduction of immunoreactivity confirms that Oatp58Dc is localized to the subperineurial, perineurial, and neural cells of the adult Drosophila brain.

RNAi constructs, expression of oatp58Dc was significantly reduced: −19.98-fold (n = 5, P < 0.05) for repo K/D 39470 and −5.17-fold (n = 4, P < 0.05) for repo K/D 39469. The loss-of-function allele oatp10642 is usually lethal when homozygous. However, 1 in 10 adult flies survived as homozygotes, and the survivors were used for qRT-PCR analysis. A significant reduction of mRNA expression was detected for oatp10642 (−32.44-fold, n = 5). This confirms that the transgenic and mutant alleles used in our studies modify the expression of Oatp58Dc in the predicted way.

Oatp58Dc is important in maintaining a chemical barrier against fluorescein influx into the brain. To assess whether the Oatp58Dc transporter is involved in maintaining a functional barrier against fluorescein influx into the brain, flies were injected with 300 μM fluorescein in saline, and fluorescein content was measured in the heads and bodies. Injected flies were left to recover for 15 min before the heads were severed from the bodies. For each genotype, the heads and bodies of flies that had not been injected were analyzed to determine the background fluorescence in the samples, and this value was subtracted from each time point.

Pinsonneault et al. (44) estimated that it takes ~3 s for an injected compound to diffuse throughout the Drosophila adult hemolymph, including the head. This is approximately the duration of the injection. Therefore, 15 min is more than ample time for the fluorescein to diffuse throughout the entire body. When Oatp58Dc was knocked down in glial cells (Fig. 7A) using oatpRNAi3970/+;repoGal4/+ (repo K/D 39470) and repoGal4/oatpRNAi39469 (repo K/D 39469), there was a significant increase in fluorescein concentration in the head. The increase in fluorescein concentration in the head was also observed in loss-of-function oatp10642 flies. The corresponding bodies were also analyzed for fluorescein content (Fig. 7B). There were no significant differences in fluorescein concentration in the bodies 15 min following injection.

In the case of the RNAi knockdowns, the decrease in Oatp58Dc expression occurs only in glial cells; thus the change in efflux from the head can be attributed to changes in efflux from the brain. However, because the head contains other tissues besides the brain, we repeated this experiment using
specific drivers fluorescein to the brain. Using the perineurial- and subperineurial-gated if one of these layers was critical for preventing access of perineurial and subperineurial glia (Fig. 4). We therefore investigated the contribution of Oatp58Dc in each of these layers to the brain. However, we found that Oatp58Dc is expressed in the subperineurial glia was reduced using spg K/D 39469 (Fig. 4, A). When Oatp58Dc expression was reduced in the perineurial glia using spg K/D 39470, there was no significant increase in fluorescein penetration into the brain (Fig. 4B). No significant differences were found in the bodies when Oatp58Dc expression was knocked down (Fig. 4, C and D). Together, the results indicate that expression of Oatp58Dc in the perineurial glia is necessary to prevent movement of fluorescein into the brain.

### DISCUSSION

The BBB protects the CNS by maintaining a homeostatic environment for neurons and plays a neuroprotective role in preventing access of potentially harmful compounds to the CNS. This highly effective barrier is the result of a tight diffusion barrier and transcellular ion transporters. More than 500 transporters have been found at the mouse BBB (13), and similar diversity is likely at the human or rat BBB. In Drosophila, the SLC21/Oatp family has eight putative Oatp transporters. Because of the complexity of the vertebrate BBB, understanding the physiological contribution of individual transporters or families of transporters remains a challenge. We chose to approach this challenge using the Drosophila glial-derived BBB, which isolates the CNS from an open circulatory system.

Our results show that, in addition to the known paracellular movement of fluorescein across the BBB, ion transporters are important in fluorescein efflux from the Drosophila brain. We have also identified Oatp58Dc as important in regulating fluorescein efflux from the brain by excluding it at the level of the BBB. These studies suggest that Oatp58Dc, a member of the SLC21 family, contributes significantly to maintaining an effective chemical protection barrier against penetration of small organic anions into the CNS.

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**Fig. 7.** Fluorescein concentration in the head and body were measured 15 min following injection in flies with reduced expression of Oatp58Dc transcript. A: fluorescein concentration in the head was significantly higher when Oatp58Dc expression was specifically reduced in glial cells using repo Gal4 or ubiquitously reduced using oatpRNAi3942. B: no significant differences were found in fluorescein concentration in the fly bodies. Values are means ± SE; n = 5 for each genotype. *P < 0.05 (by 1-way ANOVA).

**Fig. 8.** Fluorescein concentration in dissected brains 15 min following injection in flies with reduced Oatp58Dc expression in glial cells. A: fluorescein concentration was significantly higher in brains of flies with reduced Oatp58Dc expression in the BBB or ubiquitously following injection in flies with reduced expression of Oatp58Dc transcript. B: no significant differences were found in fluorescein concentration in the dissected brain tissue (Fig. 8). Results of this experiment were consistent with results of experiments that used severed whole heads. Reducing the function of Oatp58Dc expression in the BBB or ubiquitously resulted in increased fluorescein concentration in the brain (Fig. 8A), but not in the body (Fig. 8B).

*Oatp58Dc expression in the BBB is necessary to maintain a barrier against fluorescein influx into the brain.* Results expressing the glial-specific driver repo Gal4 indicate that Oatp58Dc is important in maintaining a barrier against fluorescein influx into the brain. However, we found that Oatp58Dc is expressed in the perineurial and subperineurial glia (Fig. 4). We therefore investigated if one of these layers was critical for preventing access of fluorescein to the brain. Using the perineurial- and subperineurial-specific drivers pg Gal4 and spg Gal4 (5), respectively, we investigated the contribution of Oatp58Dc in each of these layers to maintenance of a barrier against fluorescein influx into the brain (Fig. 9). When Oatp58Dc expression was reduced in the perineurial glia using pg Gal4/+; oatpRNAi39469/+ (pg K/D 39469), fluorescein penetration into the brain was significantly increased (Fig. 9A). There was a trend toward an increase when Oatp58Dc expression was reduced in the perineurial glia using pg Gal4/; oatpRNAi39470 (pg K/D 39470), but the difference was below the level of significance (P = 0.19). When Oatp58Dc expression in the subperineurial glia was reduced using spg Gal4/+; oatpRNAi39469/+ (spg K/D 39469) and spg Gal4/oatpRNAi39470 (spg K/D 39470), there was no significant increase in fluorescein penetration into the brain (Fig. 9B). No significant differences were found in the bodies when Oatp58Dc expression was knocked down (Fig. 9, C and D). Together, the results indicate...
Ion transporters are involved in fluorescein efflux from the brain. Paracellular movement of organic anions between glial cells of the subperineurial glia has been shown to be effectively limited by septate junctions that tightly cement the glial cells together (37). Septate junction formation is regulated by a G protein-coupled receptor known as Moody. Moody expression is a critical regulator of cortical actin, which stabilizes the morphology of surface glia. If there is deregulation of glial morphology by loss of Moody activity, septate junctions are unable to achieve a sufficient length to properly seal the nervous tissue (47). The result is an increase in paracellular permeability of the BBB, which allows penetration of ions that would normally be excluded, such as the small organic anion FITC, into the neural tissue (37).

When we reduced Moody expression in glial cells by expressing the moodyrNAi insert with the glial-specific driver repoGal4, fluorescein concentration in the head increased significantly, consistent with an increase in paracellular permeability of the BBB. This result validated our use of homogenized whole heads to screen potential modulators of BBB permeability. Identified modulators were then further analyzed using dissected brain tissue.

Competitive inhibition of ion transport using the nonfluorescent organic anion salicylate suggested that ion transporters play a significant role in preventing access of fluorescein to the brain. We thus aimed to identify an ion transporter that might be involved in efflux of fluorescein from the brain. Although fluorescein transport by Drosophila Malpighian (renal) tubules is predominantly Na⁺-dependent (34), there are precedents for fluorescein transport by Na⁺-independent transporters. Fluorescein uptake across the rabbit choroid plexus is maintained in the absence of Na⁺ (9), and the Na⁺-independent transporter MRP2 is implicated in fluorescein efflux across the rat BBB (29). Fluorescein is also transported by OATPB1 and OATPB3 in rat and human hepatocytes (14).

Oatp58Dc is involved in fluorescein efflux at the BBB. Of the eight putative Oatp transporters identified in Drosophila, Oatp58Dc and Oatp74D are enriched in the adult brain. On the basis of recent studies of the Malpighian tubules (10, 12), Oatp58Dc was investigated as a possible ion transporter of small type I organic anions across the BBB. Because Oatp58Dc protein is expressed in the perineurial and subperineurial glia, this transporter is in an ideal location to contribute to chemical properties of the BBB. Three independent oatp58Dc lines were used to test the effects of reduced Oatp58Dc expression on fluorescein efflux. We used two independent tissue-specific knockdowns of Oatp58Dc transcript and a ubiquitous loss-of-function allele for oatp58Dc. In all three lines, a reduction of Oatp58Dc expression in the BBB is associated with an increase in penetration of the small type I organic anion fluorescein into the brain. This was first seen by whole head analysis and further confirmed with dissected brain tissue.

Oatp transporters move substrates down their concentration gradients. Location of Oatp58Dc on the luminal side of the BBB would facilitate uptake of fluorescein into the brain. We suggest that Oatp58Dc may be located on the abluminal (basolateral) membrane of the BBB, which would result in influx of fluorescein from the brain into the BBB, consistent with other Oatp transporters. In rodents, Oat1a5 and Oat3 are localized primarily on the abluminal membrane, while Oat1a4 is localized on the abluminal and luminal (apical) membranes (19, 39, 40, 45) and Oat1c1 is expressed in the basolateral membrane of the choroid plexus (56). In Drosophila,
Oatp58Dc has been shown to be localized to the basolateral membrane of Malpighian tubules (57), consistent with an abluminal expression in the BBB. An ATP-dependent transporter located on the apical membrane would then transport fluorescein out of the BBB into the blood (39). However, according to our results, it is possible that Oatp58Dc acts to extrude organic anions from the brain that permeate the BBB from the hemolymph and may not reduce the overall penetration of organic anions into the brain.

To tease apart the contribution of Oatp58Dc to fluorescein efflux in the two layers of the BBB, we separately targeted expression of the RNAi knockdown inserts to the perineurial and subperineurial glia. Knockdown of Oatp58Dc expression in the perineurial glia resulted in a significant increase in fluorescein penetration into the brain. However, knockdown of Oatp58Dc expression in the subperineurial glia did not enhance penetration of fluorescein into the brain. This suggests that the perineurial barrier is necessary to maintain a chemical barrier against fluorescein influx. However, it is possible that Oatp58Dc is not strongly expressed in the subperineurial glia, as the colocalization is not as strong in some areas as others. In addition, the spgGal4 driver may not be as effective as pgGal4 in driving expression of the RNAi constructs to reduce Oatp58Dc expression. Until now, there has been no known function for the perineurial glial cells in organic ion transport, although the perineurium has a critical role in maintaining inorganic ion homeostasis (27). Our studies provide the first evidence for both expression and a physiological function of an organic ion transporter in the perineurial glia.

Although fluorescein is known to be transported by human OATP1B1 and OATP1B3 (14, 22), this research is the first to our knowledge that shows, in vivo, that ion transporters are involved in fluorescein efflux from the Drosophila BBB. It is also the first to our knowledge to identify a physiological function for Oatp58Dc in the Drosophila BBB and identifies a function for perineurial glial cells in chemoprotection of the brain. Future studies on Oatp transporters in the BBB should aim to address whether other Oatp transporters in the BBB contribute to fluorescein efflux. Oatp74D is enriched in the brain (http://flyatlas.org/), and Torrie et al. (57) showed, in addition to Oatp58Dc, expression of Oatp74D, Oatp30B, and Oatp33Ea in the adult head. It is also of interest to determine whether compensatory mechanisms alter the phenotype of the BBB in response to loss of Oatp58Dc or other transporter function. In addition, dynamic changes in expression, indicative of phenotypic plasticity, have been observed for Oats when flies are reared on diets enriched in organic anions such as salicylate (11, 46). Future studies should be directed at phenotypic plasticity of ion transporters in the BBB. Understanding the plasticity of these transporters, especially under prolonged dietary exposure to organic ions in Drosophila, may help us understand and clarify the role of the BBB in changes in sensitivity after repeated or prolonged exposure to neuroactive drugs (3, 4, 60).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.S. and M.J.O. are responsible for conception and design of the research; S.S. performed the experiments; S.S. analyzed the data; S.S. and M.J.O. interpreted the results of the experiments; S.S. prepared the figures; S.S. drafted the manuscript; S.S. and M.J.O. edited and revised the manuscript; M.J.O. approved the final version of the manuscript.

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