Supplemental figure titles and legends:

Figure S1. (Related to Figure 1) PER in a subset of BBB regulates circadian xenobiotic permeability, but not circadian behavior. a) RHB does not vary in bodies across the day. WT flies were anesthetized with CO2 and injected with RHB. After 60 mins of recovery, fly bodies were homogenized in PBS. The supernatant was collected and measured using a fluorescence reader at Ex540/Em595. Individual data points and means are shown. n=14-21 per time point from 3 experiments (WT). b) Daunorubicin also shows a nighttime increase in BBB permeability. WT Iso31 flies were injected with daunorubicin. After 60 mins of recovery, fly brains were dissected and dissociated in groups of 5. The corresponding bodies were homogenized and the supernatant was measured using a fluorescence reader at Ex490/Em520. n=5-6 per time point, pooled from 2 experiments. c) PG glia greatly outnumber SPG in the BBB. Whole brains of SPG-*GAL4>UAS-nGFP* or PG-GAL4>*UAS-nGFP* were imaged using a confocal microscope. Images of representative Z-stacks are shown. d) PER localizes to PG, but not SPG. Brains from SPG-*GAL4>UAS-nGFP* or PG-*GAL4>UAS-nGFP* were stained with anti-PER antibody at ZT2. Representative images of brain surface are shown. e) Loss of the clock in the BBB does not affect behavioral rhythms. Iso31 or PG-*GAL4>UAS-dnCyc* flies were singly housed in glass tubes within *Drosophila* activity monitors for 3 days in 12:12 LD and 5 days in DD. Representative actograms are shown where black lines represent the activity of a fly crossing an infrared beam within the tube. n=16. (f-g) RHB permeability in *GAL4* controls. (f) PG-*GAL4* and (g) SPG-*GAL4* were injected with RHB under CO_2 anesthesia and assessed by Ex540/Em595 fluorescence after 60 mins. Means ± SEM of the ratio of brain:body fluorescence are depicted. n=17-29, pooled from 4 experiments. pCycle indicates a presence of rhythm using JTK cycle analysis.

Figure S2. (Related to Figure 2) Neither influx of RHB nor efflux transporter expression is rhythmic in the fly brain. a) Brains from microwaved and live flies were dissected and incubated with RHB. Dye was washed off and immediate imaged with a confocal microscope. Initial fluorescence is shown as means ± SEM. n=3 from one representative experiment (left panel). Initial fluorescence from

live brains assayed at ZT4-8 or ZT12-16 is shown as means ± SEM. n=6-7 pooled from 3 independent experiments (right panel). b) Expression of Mdr65 and Mdr49 mRNA in brains was determined by qPCR and normalized to actin. c) Brains collected at different circadian times were incubated with c219 anti-pgp antibody and imaged with a confocal microscope. Representative images are shown; n=8 from 2 independent experiments.

Figure S3. Related to Figure 3. Knock-down of Inx1 in glia affects RHB permeability. a) Limited numbers of BBB glia can be obtained from *Drosophila* heads. Heads from *repo-GAL4>UAS-mCD8GFP* (glia) flies, or brains from SPG-*GAL4>UAS-mCD8GFP* (SPG) or PG-*GAL4>UAS-mCD8GFP* (PG) flies were dissociated and sorted by FACS. Representative sorting gates and percentage of live cells are shown. b) Expression of *Inx1* and *Inx2* mRNA. RNA was extracted from sorted total brain cells, sorted glia, sorted SPG, and sorted PG and examined for *Inx1* and *Inx2* enrichment. n=1 (sorted brain), n=3 (glia, SPG, PG). Means ± S.E.M. are shown. c) Effect of RNAi knockdown on *Inx1* expression. *Repo-GAL4*, *UAS-Inx1*^{RNAi} and *Repo>Inx1*^{RNAi} flies were analyzed for *Inx1* mRNA levels by qPCR. d) Permeability rhythm is affected by knockdown of *Inx1* in PG cells. PG-*GAL4>UAS-Inx1*^{RNAi} (TRiP) flies were injected with RHB under at different time points and the levels of RHB in individual fly brains and bodies were measured using a fluorescence reader at Ex540/Em595 after 1 hr. The ratio of RHB in the brain compared to bodies is shown. n=10-22, pooled from 3 independent experiments. pCycle indicates a presence of rhythm using JTK cycle analysis. (e-f) Innexin manipulations do not affect septate junction localization or function. e) Brains from wild-type, PG-*GAL4>UAS-Inx1*^{RNAi}, PG-*GAL4>UAS-*dnInx2, and SPG-*GAL4>UAS-dnInx2* were incubated with anti-DLG1 antibody to localize septate junctions. Representative images from the posterior surface are shown. f) Iso31 wild-type, PG-*GAL4>UAS-Inx1*^{RNAi}, PG-*GAL4>UAS-dnInx2*, SPG-*GAL4>UAS-dnInx2*, and NrxIV^{mut} flies were injected with fluorescently labeled 10kd Dextran (either conjugated with TMRD (red) or AF647 (white). Although TMRD gave a better signal, it could not be used with dnINX2, which is tagged with RFP. 3/16 of the NrxIV^{mut} flies showed a leaky phenotype. All other lines had no leaky brains. n=16-20. Representative images are shown.

Figure S4. (Related to Figure 4) Intracellular magnesium and calcium concentrations in the BBB. a) $[Mg^{2+}]i$ levels in *Drosophila* brains were measured by flow cytometry. WT brains were dissected in AHL, incubated with DMSO, Magfura2, Magfura2 + ionophore A23187 at room temperature, washed with AHL, and dissociated. Cells were then analyzed by flow cytometer. Fluorescence intensity of all cells is shown as overlaid histograms from a representative experiment. b) Brains from *9-137-GAL4>UAS-mCD8GFP* $[Mg^{2+}]i$ dissected at ZT2, ZT8, ZT14, or ZT20, were incubated with Magfura2-AM and measured by flow cytometry. The percent change in mean fluorescence intensity (MFI) of Magfura2 within the GFP+ population is shown as means ± SEM. n = 4 from 4 independent experiments. pCycle indicates a presence of rhythm using JTK cycle analysis. c) Brains from *9-137-GAL4>CaLexA* flies were dissected at ZT2, ZT8, ZT14, or ZT20 and imaged with a confocal microscope. Means ± SEM of fluorescence are shown. n=11-14, pooled from 2 experiments. pCycle indicates a presence of rhythm using JTK cycle analysis. d) Cal630-AM was used to measure $[Ca^{2+}]i$ in SPG-*GAL4>UAS-mCD8RFP* brains dissected at ZT2, ZT8, ZT14, or ZT20. Brains were incubated with Cal630-AM, washed, dissociated, and analyzed by flow cytometry. The percent change in MFI of Cal630 within the RFP+ population is shown as means ± SEM. n = 6 from 4 experiments.

Figure S5. (Related to Figure 5) *Eas* mutants exhibit comparable intake of phenytoin throughout the day. Flies were starved for 24 hrs and given agar-sucrose with Blue 1 and phenytoin for 2 hrs throughout the day. Flies were homogenized in PBS. The supernatant was collected and absorption measured at 260nm. Shown are individual data points ± S.D. n=12 from 2 independent experiments.

STAR Methods:

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Amita Sehgal (amita@pennmedicine.upenn.edu).

Experimental Model and Subject Details

Drosophila lines

Fly stocks were raised and maintained on cornmeal-molasses medium at 25℃. The w^{1118} iso31 strain was used as wild type. When tested as controls, UAS and GAL4 fly lines were tested as heterozygotes after crossing to iso31. 5-14 day old adult female flies were used for experiments; the age range is further specified for each method.

Method details

Permeability in fly brains

5-7 day old adult female flies were entrained to 12:12 LD cycles. RHB and daunorubicin delivery methods are similar to those previously described (Bainton et al., 2005). Briefly, a microinjection needle delivered intrahumoral 2.5 mg/mL RHB or 9 mg/ml daunorubicin in PBS between the posterior abdominal wall body segments of CO_2 anesthetized flies. Capillary action or positive pressure was applied to the needle under direct visualization over 1-2 secs to deliver an average volume of 50 nL per injection. Flies were given 1 hr after injection to rest and efflux the RHB at 25℃. Brains from the f lies were rapidly dissected in 1X PBS and washed with PBS before being placed in 50 µL 0.1% SDS. Brains were dissociated over >30 mins and the dye from brain samples was measured at excitation/emission: 540/590 using a Victor 3V (Perkin Elmer) plate reader. Bodies from the corresponding flies were homogenized, spun down and measured at the same wavelengths. Uninjected flies were used to adjust for background and the ratios of brain to body levels were calculated for individual flies for RHB and from 5 flies pooled for daunorubicin. Animals with less than 0.1 mg RHB

644 detected in the body were excluded from analysis due to increased variability of the brain:body ratio at
645 brain RHB detection threshold. Amount of RHB was calculated using a standard curve.
646
647 *Live imaging of fly brains RHB efflux*
648 Iso31 female flies were entrained to 12:12 LD cycles. Brains were carefully dissected in minimal
649 hemolymph solution HL3.1 (Feng et al., 2004) (70mM NaCl, 5mM KCl, 4 $MgCl_2$mM, 10mM $NaHCO_3$,
650 5mM trehalose, 115 sucrose, and 5mM HEPES, pH 7.4) with forceps with care to minimize damage to
651 the surface of the brain. Brains were incubated in RHB (125 µg/mL) with or without verapamil (100
652 µg/mL) for 2 mins and washed with HL3.1 media to image immediately. To determine the loss of
653 fluorescence due to diffusion and photobleaching during imaging, flies were microwaved for 1 min prior
654 to brain dissection. Brains were imaged for 10 mins using a Leica SP5 confocal microscope. FIJI
655 software was used for analysis.
656
657 *Immunofluorescence microscopy*
658 Fly brains were dissected in cold PBS and fixed in 4% paraformaldehyde (PFA) for 10-20 min at room
659 temperature. Brains were rinsed 3 × 10 min with PBS with 0.1% Triton-X (PBST), blocked for 30–
660 60 min in 5% normal donkey serum or goat serum in PBST (NDST), and incubated overnight at 4℃ in
661 primary antibody diluted in NDST. Brains were then rinsed 4 × 10 min in PBST, incubated 2 hrs in
662 secondary antibody diluted in NDST, rinsed 4 × 10 min in PBST, and mounted with Vectashield.
663 Primary antibodies included guinea pig anti-PER UP1140 (1:1000), mouse anti-PGP C219 (10 µg/ml),
664 and guinea pig anti-INX2 (1:1000), and guinea pig mouse anti-DLG1 (1:50). Secondary antibodies
665 included goat anti-guinea pig Cy5 (Rockland), goat anti-guinea pig anti-AF555 (Jackson Immuno),
666 donkey anti-mouse AF647 (Jackson Immuno). Brains were imaged using a Leica SP5 confocal
667 microscope. FIJI software was used for analysis.
668

For dextran-dye penetration experiments, we injected fluorescence tagged 10000 molecular weight fixable dextran (25 mg/ml TMRD or 10 mg/ml AF647) into fly hemolymph under CO_2 anesthesia as previously described (Pinsonneault et al., 2011). 16-24 hours after injection, flies were anesthesized using CO_2 and decapitated. The proboscis was quickly removed from the head and the head was immediately submerged in 4% PFA for 15 min. Following fixation, the brain was dissected and washed for < 4 × 10 min in PBST and mounted in Vectashield. Brains were imaged using a Leica SP5 confocal microscope.

Rest:activity rhythms assays

Locomotor activity assays were performed with the *Drosophila* Activity Monitoring System (Trikinetics) as described previously (Williams et al., 2001). 5-7-day-old female flies were entrained to a 12:12 LD cycle for 3 days then transferred to constant darkness for 5 days. Flies were maintained at 25℃ throughout the assay.

FACS sorting glial populations

Brains were dissected in ice cold HL3.1 and were maintained on ice except during dissociation. Collagenase A and DNase I were added to final concentrations of 2 mg/mL and 20 µl/mL, respectively. Brains were dissociated at 37℃ using a shaker at 250 rpm for 20 mins pausing at 10 mins to pipette vigorously. Dissociated tissue was filtered through 100 µm cell strainer and sorted using a 100 µm nozzle on a BD FACSAria (BD Biosciences). Dead cells were excluded with 4,6-diamidino-2-phenylindole (DAPI). Doublets were excluded using FSC-H by FSC-W and SSC-H by SSC-W parameters. GFP^+ cells gates were set using according to GFP^- brain tissue. Data were analyzed using FlowJo version 10.3 (Tree Star).

Quantitative PCR

Brains were dissected in cold PBS and immediately lysed. RNA was extracted using RNeasy mini kit and reverse transcribed to cDNA using random hexamers and Superscript II (Invitrogen). Real-time polymerase chain reaction (PCR) was performed using Sybr Green PCR Master Mix (Applied Biosystems) with the oligonucleotides described in Table 2. Assays were run on ViiA7 Real-Time PCR system (Applied Biosystems). Relative gene expression was calculated using the ΔΔCt method normalizing to actin.

Flow cytometric assay for intracellular magnesium and calcium levels

Brains from entrained adult female flies (5-7 days) with fluorescently-labeled PG or SPG were dissected in adult hemolymph-like saline (AHL; 108mM NaCl, 5mM KCl, 2mM $CaCl_2$, 8.2mM $MgCl_2$, 4mM $NaHCO_3$, 1mM NaH_2PO_4-H_2O, 5mM trehalose, 10mM sucrose, 5mM HEPES; pH 7.5) on ice. Brains were brought to room temperature (RT) for 10 mins and incubated with 5 µM Magfura2-AM and 5 µM Cal630-AM for 20 mins at RT. Brains were washed with RT AHL for 3 x 5 mins. Then Collagenase IV and DNase I were added to final concentrations of 2 mg/mL and 20 units, respectively and brains were dissociated at 37˚C with 250 rpm shaking for 15 mins. Dissociated tissue was filtered through 100 µm cell strainer and washed with FACS buffer (PBS with 1% w/v bovine serum albumin and 0.1% w/v sodium azide). Cells were analyzed on BD FACSCanto II (BD Biosciences). Doublets were excluded using FSC-H by FSC-W and SSC-H by SSC-W parameters. RFP^+ cells gates were set according to RFP^- brain tissue. Data were analyzed using FlowJo version 10.3.

Seizure recovery assay

7-14 day old adult female eas^{PC80} flies were starved for 24 hrs to allow for maximum drug dosing. Flies were given 5% sucrose and 1.5% agar with or without 0.6 mg/mL phenytoin a previously described dose to improve recovery from seizure (Reynolds et al., 2004). Flies were tested in 2 vials (1 control and 1 phenytoin), each containing 15 flies. Mechanical shock was delivered by vortexing flies at high speed for 5 secs. The assays were video recorded and the number of flies seizing was recorded at 15

sec intervals until the entire population had recovered. Recovery was defined as standing and was analyzed by a researcher blinded to the drug condition. Mean recovery time was calculated as the average time it took any individual fly to recover in a population. Flies that never seized were calculated as 0 secs for recovery time.

The blue dye feeding assay was performed as previously described (Deshpande et al., 2014). Briefly, after 24 hrs of starvation, flies were given food with 2% w/v FD&C Blue No. 1 for 2 hrs. Individual flies were homogenized and the absorbance at 620 nm was measured with a Victor 3V (Perkin Elmer) plate reader. The amount of food eaten was calculated by using a standard curve.

Quantification and Statistical Analysis

The statistical details of experiments can be found in the figure legends. Circadian statistical analysis was performed in R using JTK_CYCLEv3.1. ANOVA and post-hoc Tukey tests were performed with GraphPad Prism. Student's T-tests were performed with Excel. Sample sizes were determined with powerandsamplesize.com.