Supporting Information

Enantioselectivity of 2,2',3,5',6-pentachlorobiphenyl (PCB 95) atropisomers toward ryanodine receptors (RyRs) and their influences on hippocampal neuronal networks

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MATERIALS AND METHODS

Preparation of RyR1-enriched junctional sarcoplasmic reticulum (*JSR*).

All procedures with animals were approved by the UC Davis IACUC Committee. For macroscopic Ca^{2+} flux measurements, a sucrose-gradient purified junctional SR (JSR) membrane vesicles fraction enriched in RyR1 and SR/ER Ca^{2+} ATPase (SERCA) was isolated from fast-twitch skeletal muscles of <1 year-old male (~3 kg) New Zealand White rabbits, as previously described [1, 2]. The preparations were flash frozen and stored in 10% sucrose, 10 mM HEPES, pH 7.4, at −80 °C until needed. Functional experiments reported in this study were performed on two JSR preparations isolated on different days.

Preparation of mouse skeletal muscle, cardiac muscle and brain cortex homogenates.

Direct comparison of whether *(aR)-, (aS)-* and *rac*-PCB 95 differ in activity towards preparations that expressing defined RyR isoform(s) were performed using the following tissues from C57 BL/6 mice ranging in age from 3 to 6 months: Skeletal muscle (a pure source of RyR1), heart ventricles (a pure source of RyR2), and brain cortex (a mixture of RyR1, RyR2 and RyR3)[3-5]. Mouse tissues were flash frozen in liquid nitrogen and finely ground using a mortar containing liquid nitrogen. The ground tissues were then added to ice-cold homogenization buffer composed of (in mM) 300 sucrose, 5 imidazole, 0.1 phenylmethanesulfonylfluoride (PMSF), and 10 µg/ml leupeptin. To obtain microsomal homogenates of skeletal and cardiac muscle, tissue was placed in ~1:20 (v/v) buffer and subjected to two sequential bursts 30s each at 23,000 rpm using a PowerGen 700D (Fisher). The homogenates were first centrifuged at 1,000 rpm for 60

sec at 4^{o}C, the pellets were discarded, and the supernatants were poured through three layers of cheesecloth. The filtrates were centrifuged at 110,000 × g for 60 min at 4 °C, pellets were then re-suspended in 300 mM sucrose, 10 mM Hepes, pH 7.4, aliquoted into microcentrifuge tubes (300 µl/sample), and stored at −80 °C for biochemical analyses. To obtain brain cortical microsomes, the finely ground tissue was suspended in the buffer described above at ~1:50 (v/v) and homogenized using a Potter-Elvehjem tissue grinder for 30 s, repeated 3 times, 30 s intervals between two homogenization. Homogenates were then centrifuged at 1000 rpm for 30 s, supernatant were collected and further centrifuged 110,000 x g for 1 h at 4 ^{o}C. The pellets were re-suspended in 300 mM sucrose, 10 mM Hepes, pH 7.4, aliquoted into microcentrifuge tubes and flash-frozen in liquid nitrogen and stored at – 80^{o}C until needed. Biochemical studied reported from these homogenates were obtained from at least two independent microsomal preparations on separate days.

Determination of protein concentrations.

Protein concentration of each membrane preparation was determined using the DC protein assay kit (Bio-Rad, Richmond, CA).

Measurements of [^{3}H]Ryanodine ([^{3}H]Ry) *Binding.*

Equilibrium binding of [^{3}H]Ry to the skeletal muscle(0.05 mg/mL for JSR, 0.25mg/mL for homogenate), cardiac muscle (0.25mg/mL) and brain cortex homogenates (0.4 mg/ml) was measured at 37°C for 3 h with constant shaking in binding buffer consisting of 1nM (JSR), 2 nM (skeletal muscle and cardiac muscle) or 10 nM (brain cortex) [^{3}H]Ry (in mM) 250 KCl, 14 NaCl, 20 HEPES, pH 7.4, and 2 µM free Ca^{2+} (obtained by the addition of EGTA calculated according to the software

Bound-and-Determined [6]. Nonspecific [^{3}H]Ry binding was measured as the residual binding measured in the presence of a 1,000-fold excess of unlabeled ryanodine. Bound and free ligand were separated by rapid filtration through Whatman GF/B glass fiber filters (Whatman, Gaithersburg, MD) using a 48-sample Cell Harvester (Brandel, Gaithersburg, MD) and three washes with 5 ml of ice-cold buffer (in mM) 250 KCl, 14 NaCl, 20 HEPES, and 2 μM Ca^{2+} pH 7.4). [^{3}H]Ry retained on filters was quantified by liquid scintillation spectrometry using a Beckman model 6500 scintillation counter. Each radioligand-receptor binding experiment was performed on at least two independent membrane preparations, each in quadruplet or triplicate.

Measurements of Ca^{2+} flux and Ca^{2+}-induced Ca^{2+} release.

The active uptake and passive release of Ca^{2+} from JSR membrane vesicles was measured in real-time using the Ca^{2+} sensitive metallochromic dye Arsenazo III as previously described using dye Antipyrylazo III [7]. Briefly, JSR membrane preparation was diluted to 100 μg/mL in 1500 μl assay buffer consisting of (in mM) 100 KCl, 6 sodium pyrophosphate ($Na_4P_2O_7$), 2 Mg-ATP; 10 phosphocreatine, 0.25 Arsenazo III, 20 MOPS, pH=7.4, and 20 μg/mL creatine phosphokinase to regenerate ATP. Ca^{2+} uptake into JSR vesicles was accomplished by four sequential additions of 45 nmole of $CaCl_2$. Vesicles were then exposed to either the DMSO vehicle (control) or racemic or enantiomeric PCB 95 to study passive release of accumulated vesicular Ca^{2+}, and the potentiation of Ca^{2+}-induced Ca^{2+}-release was investigated in the presence or absence of nanomolar racemic or enantiomeric PCB95. All Ca^{2+} flux measurements were performed at 35°C. Changes in Ca^{2+} concentration were obtained by measuring the Ca^{2+}-Arsenazo III absorbance at 650nm and background correction at a single

reference of 700nm using an Agilent 8453 UV-visible Spectroscopy System (Agilent Technologies, CA) and calibrated against serial dilution of a $CaCl_2$ stock certified by the National Bureau of Standards.

Hippocampal neuronal cell culture.

Hippocampal cultures were obtained from postnatal day 0-1 (P0-P1) C57 BL6 male pups. After pups were decapitated, their brains were removed and hippocampi were dissected in a sterile hood and incubated for 30 min at 37 °C in a solution of 0.25% Trypsin-EDTA (Gibco™, ThermoFisher). Hippocampi were then washed in a Krebs solution containing 0.6 mg/mL of Trypsin-inhibitor and 80 µg/mL DNase I (Sigma, USA) before dissociating the cells with a fire-polished Pasteur pipette. After non-dissociated tissue settled, cells in the supernatant were collected and centrifuged (7 min, 1400 rpm) and re-suspended in Neurobasal medium (Gibco™, ThermoFisher, USA) containing 2% GS21™ neural supplement (MTI-GlobalStem,USA), 0.5 mM Glutamax (Gibco™, ThermoFisher) and 5% Fetal Bovine Serum (Gibco™, ThermoFisher). Cells were counted and plated onto a 0.1% (w/v) poly-L-lysine-coated 96-well imaging plates (Falcon Corning) at a density of 5 x 10^4 cells/well. After 2 h, an equal volume of free-serum medium was added to the wells. Cytosine *β*-D-arabinofuranoside (Ara-C, 10µM) was added at 2 DIV (days *in vitro*) to inhibit the proliferation of astrocytes. The medium was then changed twice a week by replacing half the volume of culture medium with serum free Neurobasal supplemented medium in the presence or absence of test compounds. The neuronal cultures were maintained in an incubator at 37 °C with 5% CO_2. For the chronic study, either *rac*-, (*aR*)-, or (*aS*)-PCB 95 (50 or 200 nM final concentration), or 0.2% DMSO vehicle, was added at the same time as Ara-C and kept

in the media until 14 DIV when functional outcomes were measured from the mature neuronal networks.

Calcium imaging.

Spontaneous and electrically evoked Ca^{2+} transients were measured on dissociated hippocampal neuronal networks cultured on clear-bottom 96-well imaging plates at 14 DIV. Hippocampal neurons were loaded with the Ca^{2+}-sensitive dye Fluo-4 AM (5 μM; Invitrogen) at 37°C for 30 min in imaging buffer consisting of (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.4, and supplemented with 0.05% BSA. For the cells subjected to the Chronic Study, either *rac*-, (*aR*)-, or (*aS*)-PCB 95 (50 or 200 nM final concentration), or 0.2% DMSO vehicle was added to the loading buffer described above. Cultures were washed three times with imaging buffer and transferred to the stage of an inverted Olympus IX70 microscope (Olympus America, Center Valley, PA) equipped with a 40x 1.25 NA objective. Fluo-4 was excited at 494 nm using a DeltaRam illuminator (Photon Technologies Int, Birmingham, NJ); fluorescence emission was captured at 510 nm. Full-frame images were captured with an Evolve® cooled CCD camera (Photometrics, Tucson, AZ) at 30 frames/sec (fps) using EasyRatioPro software (Photon Technologies). After 10 min of baseline recording, cultures were stimulated with electrical bipolar field pulses (0.5 ms) at 20 Hz for 2 s using platinum electrodes connected to a Master 8 stimulator (AMPI, Jerusalem, Israel). In addition, the acute study was undertaken. Neuronal cultures naïve to PCB 95 were loaded with Fluo-4 on 14 DIV and imaged as described above for the chronic study. Following baseline recordings and a train of electrical stimulation, the cells were exposed to 500 nM *rac*-, (*aR*)-, or (*aS*)-PCB 95, or 0.5% DMSO (in the acute study),

additional baseline activity and responses to electrical stimulation were then recorded. After acquisition, regions of interest were drawn freehand to encompass soma. Movies were replayed to quantitatively measure changes in Fluo-4 fluorescence within the regions of interest.

Western blot analysis of expressed RyRs in brain cortical preps

Mouse brain cortical preparations (two preps), JSR (one prep) and cardiac microsomal prep were denatured in SDS-PAGE sample buffer (Bio-Rad Laboratories) containing 125mM Dithiothreitol (DTT) at 95°C for 5 min. Protein of 80 µg/lane (JSR, 0.3µg/lane; Cardiac 2µg/lane) was loaded onto Mini-PROTEAN Tris-Tricine precast gels gels (Bio-Rad Laboratories), electrophoresed at 150 V for 45 min at room temperature (RT), and then transferred to methanol activated polyvinylidene difluoride membranes at 400mA for 2 hr (4°C). Membranes were then blocked with Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) with 0.1% Tween-20 for 1 h at room temperature and incubated overnight at 4 ℃ plus 30min at RT with primary antibodies: monoclonal RyR1(34C) (1/200; DSHB, Reno, NA), monoclonal RyR2 (C3-33) (1/200; Abcam), polyclonal anti-RyR3 (1/100; EMD Millipore), β-actin (1/500; Cell Signaling Technology, Danvers, MA) in blocking buffer and then washed with TBS (Bio-Rad Laboratories) with 0.1% Tween-20. After washing, membranes were incubated with secondary antibodies (IRDye 680 nm and 800 nm, 1/5000, Li-Cor Biosciences) in blocking buffer for 1 h at RT, then washed again with TBS (Bio-Rad Laboratories) with 0.1% Tween-20 and developed with the Odyssey Imaging System (Li-CorBiosciences).

Data Analysis.

Radioligand receptor binding data were fitted by non-linear curve fitting using Origin® software (Northampton, MA) or Graph Pad Prism software (Version 7.03; GraphPad Software Inc., San Diego, CA).

Boltzmann function was used for sigmoid curve fitting for Figure 2. The equation is described as:

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_o)/\Delta x}} + A_2$$

Where y is the variable corresponds to the bound [^{3}H]ryanodine (f-pmole/mg protein); A_1 = initial value (Bound [^{3}H]ryanodine/protein); A_2 = final value (Bound [^{3}H]ryanodine/protein); X = the independent variable (total concentration of PCB95); X_o = center; ΔX = slope at X_o; EC_{50} = (X_o, (A_1+A_2)/2).

For Figure 3, potency values were determined by nonlinear regression with three-parameter equation using Prism Graph Pad 7.03. The equation used is followed as:

$$y = \frac{A_1 - A_2}{1 + 10^{\log EC_{50} - X}} + A_2$$

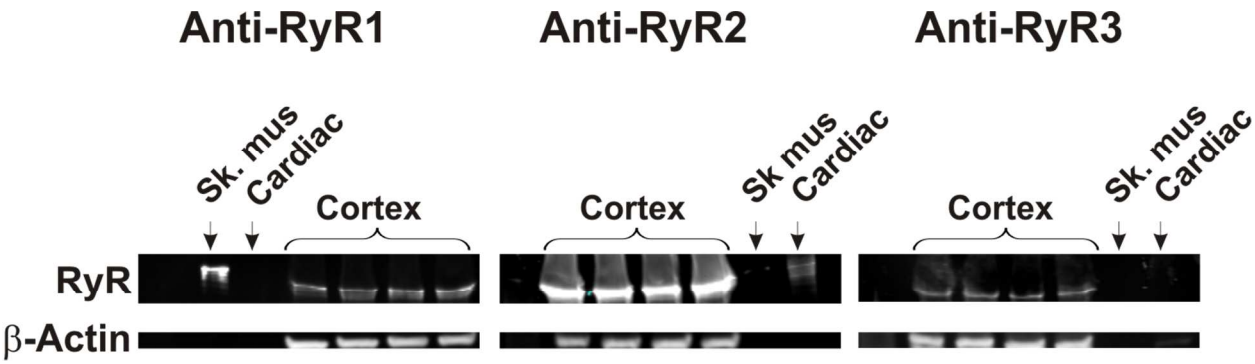
Where,

The y represents the Response, which increase as X increases; X represents the log of dose or concentration; A_1=Maximum (Bound [^{3}H]ryanodine/protein); A_2= Minimum (Bound [^{3}H]ryanodine/protein); logEC_{50}: same log unit as X

For [^{3}H]Ryanodine binding analyses compared at certain concentrations and Ca^{2+} flux measurements, One-way ANOVA followed by post hoc Dunnett's test was

used to analyze the data at 95% confidence intervals. For JSR Ca^{2+}-induced Ca^{2+} release measurements, unpaired T-test was used.

RESULTS



SI_Figure 1. Western analysis of reveals all three isoforms of RyR expressed in mouse brain cortex, with RyR2 being the most abundantly detected isoform. “Sk.mus” is rabbit skeletal muscle JSR, loaded at 0.3μg/lane; Cardiac is mouse cardiac microsomal protein 2μg/lane; cortex (mouse) is 80μg/lane.

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