1 Agonist and antagonist diverted twisting 2 motions of single TRPV1 channel

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23 **Abstract**

24 **Transient receptor potential vanilloid type 1 (TRPV1) channels are**
 25 **activated by heat, vanilloids, and extracellular protons. Cryo-EM**
 26 **has revealed various conformations of TRPV1, and these**
 27 **structures suggest an intramolecular twisting motion in response**
 28 **to ligand binding. However, limited experimental data support this**
 29 **observation. Here we analyzed the intramolecular motion of TRPV1**
 30 **using diffracted X-ray tracking (DXT). DXT analyzes trajectories of**
 31 **Laue spots generated from attached gold nanocrystals, and**
 32 **provides picometer spatial and microsecond time scale information**
 33 **about intramolecular motion. We observed that both an agonist and**
 34 **a competitive antagonist evoked rotating bias in TRPV1, though**
 35 **these biases were in opposing directions. Furthermore, the**
 36 **rotational bias generated by capsaicin was reversed between the**
 37 **wild type and the capsaicin-insensitive Y511A mutant. Our findings**
 38 **bolster the understanding of the mechanisms used activation and**
 39 **modulation of TRP channels, and this knowledge can be exploited**
 40 **for pharmacological usage such as inhibitor design.**

41 **Introduction**

42 Transient receptor potential (TRP) channels constitute a superfamily of nonselective
 43 cation channels, including 28 different genes in human. TRPV1 plays a key role in
 44 nociception and is activated by various stimuli, including heat, low pH, and vanilloids
 45 (1-3). Structures of TRPV1 have been determined by cryo-EM at near-atomic resolution
 46 (4, 5), and they provide a fundamental framework for TRP activation. TRPV1 is
 47 composed of a homotetramer, and each subunit includes six transmembrane segments
 48 and a pore-forming region. Transmembrane segment 5 (S5), the pore helix, and
 49 transmembrane segment 6 (S6) together form a pore in the assembled tetramer,
 50 surrounded by a voltage sensor-like (VSL) domain composed by a bundle of four
 51 transmembrane helices (S1–S4). Both the N- and C- termini are located inside the cell,
 52 and a large ankyrin repeat domain is present at the N-terminus.

53 Cryo-EM revealed conformational dynamics of TRPV1 induced by capsaicin,
 54 capsaizepine, resiniferatoxin (RTX) from *Euphorbia resinifera*, and a double knot toxin
 55 (DxTx) from Chinese bird spider venom (4, 6). Indeed, these cryo-EM data, as well as
 56 simulation studies (7, 8) suggest rotational and twisting motion may occur during the
 57 gating, but direct detection of this movement has remained technically challenging.

58 **Results**

59 **Single molecule analysis of TRPV1 using DXT**

60 Our studies used full-length human TRPV1 which was subcloned into
 61 pcDNA3.1 and tagged with (His)₆ sequence at the N-terminus, and tagged with the
 62 FLAG sequence (DYKDDDDK) at the C-terminus (FLAG-TRPV1) (Fig. S1A). The
 63 function of intracellular Ca²⁺-permeable FLAG-TRPV1 and mutant was assessed by
 64 cell-based Ca²⁺ flux assay in HEK-293 cells (Fig. S1B).

65 To understand the intramolecular motion of the TRPV1 upon gating, we used a
66 diffracted X-ray tracking (DXT) method (9). DXT uses broadband irradiation of X-rays
67 (Fig. S2), and is based on the principle of Bragg's law (10, 11). The diffraction rings
68 from the X-ray beams, also called Debye Schetter rings, broaden according to their
69 energy bandwidths, and the motion of diffraction spots were analyzed in two rotational
70 axis views, including tilting (θ) and twisting (χ) angles (Fig. 1A-C). The DXT
71 measurement was performed at BL40XU beamline in SPring-8 Japan. Data were
72 recorded with 100 microsecond (μ sec) per frame and a total measurement time was 10
73 millisecond (ms).

74 FLAG-TRPV1 was immobilized through a Ni-NTA self-assembled monolayer
75 formed on polyimide films (Fig. 1A). To provide direct visualization of the
76 intramolecular dynamics of TRPV1, the extracellular side of FLAG-TRPV1 was labeled
77 with gold nanocrystals through Au-S binding via Met-tags (MGGMGGM) introduced in
78 the sequence. AFM imaging shows the size of gold nanocrystals were ranging from 40
79 to 80 nm diameter (10). Two extracellular regions were separately labeled with gold
80 nanocrystals: the outer pore (OP) region and the VSL domain (Fig. 1D). The rotation
81 angle (χ) of the labeled position was determined relative to the immobilized cytoplasmic
82 domains when viewed from the extracellular side (Fig. 1D, lower).

83 The time-averaged mean square displacement (MSD) (12) curves were
84 obtained for both the VSL- and OP-labeled positions of TRPV1 (Fig. 1E). At the VSL
85 position, capsaicin enhanced the slope of the MSD curve. Interestingly, the slope was
86 also increased by the competitive antagonist AMG9810 (13). The diffusion constants
87 (D_χ) of capsaicin and AMG9810 obtained from the MSD curves were increased by 46%
88 and 41% over that of the control (ligand-free), respectively (Table S1). The D_χ s at the
89 OP-labeled position were also increased by capsaicin and AMG9810, but they were
90 much smaller than those observed at the VSL-label (Table S1) and the increasing rates

91 were limited to 2% and 7% of the control, respectively.

92 The probability distributions of χ were plotted as a function of time-interval
 93 (Δt) to investigate the dynamics of TRPV1 motion, known as the van Hove
 94 self-correlation function (VHC) (14). For the ligand-free state, the distribution at $\Delta t =$
 95 0.1 ms (Fig. 1F) and other time intervals (Fig. S3) fitted well with the Gaussian function.
 96 The standard deviation (σ) of the Gaussian function was increased from 0.51 to 0.55 by
 97 capsaicin (Fig. 1F). The mean μ values of the Gaussian function were very small in all
 98 conditions ($-5.4\text{E-}03$, $5.3\text{E-}05$ and $3.6\text{E-}03$ mrad for ligand-free, capsaicin, and
 99 AMG9810, respectively) and there was no bias in the shape of the distribution (Fig. 1F
 100 and S4). From the distributions using all detected trajectories, we could not find any
 101 significant rotational bias in TRPV1.

102 Analysis using lifetime classification

103 Because the velocities of Brownian particles diverge when time intervals
 104 approach zero (15, 16), and because the trajectories are not differentiable anywhere due
 105 to its discontinuities, a lifetime was introduced to classify the motion of trajectories (Fig.
 106 2A-C, and Supplementary Video1, Video2). An inverse relationship was clearly shown
 107 between the lifetime and the average step sizes at 0.1 ms intervals; the longer the
 108 lifetime, the smaller the step size (Fig. 2D).

109 We applied statistical analysis into three groups: short [lifetime (LT) < 2.5 ms],
 110 long ($2.5 \leq \text{LT} < 4$ ms), and the longest ($8 \leq \text{LT} < 10$ ms) lifetime groups. The diffusion
 111 constants D_χ of OP-label with capsaicin were obtained from the MSD curves; 9.87, 7.30
 112 and $5.04 \text{ mrad}^2 / \text{ms}$, for short, long, and the longest group, respectively (Fig. 2E and
 113 Table S2). In the short lifetime trajectories, we could not find any statistical significance
 114 in the mean μ values among the experimental conditions (Fig. 2F, upper and Fig. S5).
 115 However, the mean μ value of OP-label was decreased to -0.19 by capsaicin in the long
 116 lifetime trajectories (Fig. 2G). Subtraction of the probability density showed clearer

117 rotational bias in the long lifetime group than the short lifetime group (Fig. 2F and 2G).

118 The mean μ values plotted over time-interval Δt (denoted as mean plot)
119 represent the rotational bias generated in TRPV1 (Fig. 3). Rotation angle was
120 determined from the extracellular side, by the motion of the extracellular OP- and VSL-
121 region in contrast to the cytoplasmic domain, which was immobilized to polyimide film.
122 The motions of the short lifetime trajectories were again shown to be non-biased (Fig.
123 3A and Fig. S5). In contrast, the long lifetime trajectories showed unique rotational
124 biases to their sample conditions (Fig. 3B). We judged the biased motion to be
125 clockwise (CW), counterclockwise (CCW), and non-directed movement from the mean
126 plots. From previous studies, a CW bias is considered to be a motion toward channel
127 opening (4). The maximum rotation angles of capsaicin-induced TRPV1 were 1.10 and
128 1.55 degrees at 1 ms-intervals to the CW direction both at the OP and VSL positions,
129 respectively (Fig. 3C).

130 At the OP-label, all experimental conditions showed CW rotational bias.
131 However, the mean μ value at $\Delta t = 2.4$ ms ($\mu_{2.4}$) of ligand-free and capsaicin were
132 equivalent ($\mu_{2.4} = -0.23$ and -0.25 , respectively), while $\mu_{2.4}$ of AMG9810 was much
133 smaller ($\mu_{2.4} = -0.09$). As for the VSL-label, ligand-free and capsaicin binding also
134 showed CW rotational bias, while AMG9810 showed bias in the CCW direction ($\mu_{2.4} =$
135 0.10).

136 **Motion analysis of the slowest-moving group**

137 We also analyzed the movement of the slowest moving group using the longest
138 life filtering ($8 \leq LT \leq 10$ ms). Capsaicin-induced movement clearly showed the CW
139 bias, while the movement of the ligand-free condition was CCW biased at both the OP
140 and VSL positions (Fig. 4). This result shows that the capsaicin evoked relatively slow
141 movement in TRPV1, which may persist up to 10 ms.

142 **Motion analysis of capsaicin-insensitive mutant**

143 To confirm whether the CW bias was really associated with channel gating, we
 144 examined the motion of the capsaicin-insensitive Y511A mutant. Trajectories of gold
 145 nanocrystals at both OP- and VSL-label were also obtained from the Y511A mutant, and
 146 were similarly classified into short ($LT < 2.5$ ms) and long ($2.5 \leq LT < 4$ ms) lifetime
 147 groups. As was the case with the wild type channel, the short lifetime groups did not
 148 show any specific rotational bias (Fig. S6). As for the long lifetime groups, the
 149 rotational movements of Y511A in the ligand-free condition were non-biased (OP-label)
 150 or slightly biased toward CW (VSL-label) (upper panels of Fig. 5A). However,
 151 capsaicin evoked a strong bias toward the CCW direction in Y511A both at the OP- and
 152 VSL-label positions (lower panels of Fig. 5A). This was a big difference compared to
 153 the capsaicin-induced CW bias of the wild type. Capsaicin-induced CCW bias in the
 154 Y511A mutant resembles AMG9810-induced CCW bias in the wild type channel.
 155

156 Discussion

157 It is technically challenging to directly detect twisting motion of channels,
158 because the motion involves rapid conformational changes ranging from the
159 microsecond to millisecond time scale. They are beyond the temporal and spatial
160 resolution of most imaging-based techniques. DXT provide sub-angstrom scale
161 information of conformational dynamics as well as functional intermediates. In this
162 study, a diffusion constant of the glycine 602 at the OP-labeled position, which locates
163 17.3 Å distance from the center of the pore, was calculated to be 12.8 pm²/ms (Fig. 5B).

164 Because all measurements were performed in the closed sample chambers
165 under the open-closed equilibrium of channel, we could not detect any significant
166 differences in the rotational bias at the first analysis. By applying lifetime filtering,
167 however, we were able to extract the motions related to pore gating. The most
168 prominent difference was observed by the filter 2.5–4.0 ms (Figs. 3A and 3B), where
169 control and capsaicin showed equivalent levels of CW rotation bias, whereas AMG9810
170 was CCW-biased.

171 We compared cryo-EM structures of TRPV1 reported in the Protein Data Bank
172 (<https://www.rcsb.org>). Rotation angle was determined by the movement of glycine 602
173 for OP-label and tyrosine 463 for VSL-label, when the ankyrin repeat domains was held
174 as a fulcrum. In the comparison of apo- (PDB: 3J5P) and capsaicin-bound TRPV1
175 (PDB: 3J5R), we and others (17) found no obvious conformational change both at the
176 OP- and VSL- positions examined in this experiment (Fig. S7).

177 We further compared the structures of apo- (PDB: 3J5P) and the DkTx / RTX
178 bound TRPV1 (PDB: 3J5Q) to understand the twisting direction of helices in response
179 to the channel opening. The rotational motion generated by the DkTx / RTX was CW
180 direction both at the OP- and VSL- positions, with calculated angles of 10.6 and 7.1

181 degrees, respectively (Fig. 5B). TRPV2 (Ref Sec ID: NP_057197) and TRPV3
182 (NP_001245134) also rotate CW direction in response to ligand binding (18, 19) which
183 share 46% identity and 41% similarity in amino acid sequence to TRPV1, respectively.
184 Because the overall architectures of these channels are quite similar to each other,
185 intramolecular motions at channel opening may share similar mechanisms. In
186 considering the lack of obvious structural change at the OP- and VSL- positions by
187 capsaicin, capsaicin-induced rotational biases at these positions must be much smaller
188 than those by DkTx / RTX. Indeed, the capsaicin-induced absolute value of maximum
189 twisting angle determined by DXT was 1.55 degree at $\Delta t = 1$ ms (Fig. 3C).

190 This rotational motion associated with gating may reflect several intermediate
191 states of the channel. Cryo-EM presents three structures of TRPV1 so far; 'closed',
192 'partially open', and 'full open' states. At least two transition states exist along with the
193 pore opening pathway during activation of capsaicin (17). In the first pathway, the main
194 transition occurs from the closed state to the partially open state, by capsaicin binding.
195 After that, the second pathway for the channel opening occurs. For the motion of the
196 longest lifetime filtering group ($8 \leq LT \leq 10$ ms), the difference in the mean-plot curves
197 was obvious between the control and capsaicin (Fig. 4). Capsaicin-induced movement
198 was found to be CW, on the other hand, ligand-free was CCW biased. This delayed
199 movement may reflect a capsaicin-evoked second pathway from the partially-open to
200 the fully open state.

201 AMG9810, a competitive antagonist of TRPV1, interacts with the same
202 binding pocket as capsaicin. Thus, a fundamental question remains why only the agonist
203 causes channel opening. Indeed, AMG9810 was shown to induce CCW rotational bias
204 of TRPV1 in this study. Cryo-EM-based 3D structures and the computation studies (4, 7,
205 17) have suggested how capsaicin activates TRPV1 at the atomic level. Briefly, residues
206 Y511, T550, and E570 mainly contribute to capsaicin binding (Fig. 5B and 1D). Upon

207 binding, capsaicin is stabilized by T550 through Van der Waals interactions, followed by
208 a series of structural rearrangements generating an outward pushing force to the S4-S5
209 linker by E570 through hydrogen bonding, and opening the S6 activation gate by
210 flipping the aromatic side chain of Y511 (7). Loss of the hydroxyl group from the
211 vanillyl group in AMG9810 may abrogate the ability to interact with the S4-S5 linker,
212 while interactions with the binding pocket remains (7, 20)

213 In this study, we found that even the ligand-free state of TRPV1 has a bias
214 toward CW (pore opening) direction, which was not seen in the Y511A mutant proteins
215 (Fig. 3A-B and 5A). Considering the proposed mechanisms, a flip-flop fluctuation of
216 the side chain in Y511 may trigger the rotation movement. Indeed, this flipping motion
217 was demonstrated by MD simulation analysis in the ligand-free form (21).

218 Capsaicin evoked strong CCW (pore closing) bias to Y511A (Fig. 5A), which
219 may be in correlation with the results of AMG9810-induced CCW rotation bias in wild
220 type. A loss of interaction between Y511 and the S6 segment in the Y511A mutant
221 proteins may deflect the rotation bias to the CCW direction.

222 Determining of structural changes at single-molecule level, together with the
223 single channel patch clamp techniques, could be expected to elucidate the exact role of
224 channels such as allosteric mechanism and desensitization of TRPV1.

225

226 **Materials and Methods:**

227 **Plasmid Construct and protein purification.** Full-length human TRPV1 was
 228 subcloned into pcDNA3.1 and tagged with (His)₆ sequence at the N-terminus, and
 229 tagged with the FLAG sequence (DYKDDDDK) at the C-terminus (Fig. S2A). Met-tag
 230 (MGGMGGM) was introduced at an extracellular loop between the S1 and S2
 231 segments (for VSL-labeling), or a loop between the S5 segment and the pore-forming
 232 domain (for OP-labeling). The TRPV1 protein was expressed in HEK293F cells, and
 233 purified with two step purification by an anti-FLAG M2 affinity chromatography
 234 (Sigma) and a Superdex 200 size exclusion chromatography (GE Healthcare, IL). The
 235 peak fractions were concentrated to 0.1 mg/mL for DXT (Fig. S2C). Alanine
 236 substitution at the 511 tyrosine was made by site-directed mutagenesis using the
 237 QuikChange mutagenesis kit (Agilent Technologies).

238 **Sample preparation for DXT assays.** Gold nanocrystals were obtained via
 239 epitaxial growth on a KCl (100) surface and dissolved by a buffer containing 50 mM
 240 MOPS (pH 7.0) and 5 mM n-decyl- β -D-maltoside (Dojindo, Japan). The basement
 241 surface of the polyimide film (Du Pont-Toray, Japan) was coated with Ni-NTA
 242 self-assembled monolayer using dithiobis (C2-NTA) (Dojindo). The 20 μ L
 243 (His)₆-tagged TRPV1 (~0.1 mg/mL) was immobilized on the 50- μ m-thick polyimide
 244 film. After incubation for 6 h at 4 °C, excess protein was washed out with buffer. The
 245 gold nanocrystals, which specifically bind to the genetically introduced Met-tag
 246 (MGGMGGM) via Au-S binding, were applied. After incubation for 60 min, excess
 247 gold nanocrystals were washed out with buffer. After applying 10 μ L of phosphate
 248 buffered saline containing 10 μ M capsaicin or 10 μ M AMG9810, the sample chamber
 249 was covered with another layer of polyimide film, and sandwiched by stainless steel
 250 frames and screw-clamped. Capsaicin and AMG9810 were purchased from Sigma.

251 **DXT measurements.** X-rays from the beamline (BL40XU, SPring-8, Japan) with
 252 energy widths ranging from 14.0–16.5 keV were used for DXT measurements (Fig. S1).
 253 The beam size was adjusted to 50 μm in diameter by inserting a pinhole aperture
 254 upstream of the sample, and time-resolved diffraction images from gold nanoparticles
 255 immobilized on TRPV1 were recorded by an X-ray image intensifier (V7339P,
 256 Hamamatsu photonics) and CMOS camera (Phantom v2511, Vision Research). Data
 257 were recorded at 100 μsec /frame intervals, and measured 100 frames per spot. For
 258 each sample, diffractions at 72 spots were recorded. The distance between the sample
 259 and a detector was set to 60 mm . All DXT measurements were carried out at room
 260 temperature.

261 **Data Analysis.** The trajectories of diffraction spots on the image plane were tracked
 262 by TrackPy (v0.3.2 <https://doi.org/10.5281/zenodo.60550>) package with python. The
 263 trajectories of the diffraction spots were analyzed using custom software written within
 264 IGOR Pro (Wavemetrics, Lake Oswego, OR). Data were recorded with 100
 265 microsecond (μsec) per frame and a total measurement time was 10 millisecond (ms).
 266 Data with short-lived ($0.1 \leq \text{lifetime} \leq 0.3 \text{ ms}$) were excluded for reducing the analytical
 267 noise, because the number of short-lived data were 2.5 times of data with long-lived
 268 ($0.4 \leq \text{lifetime} \leq 10 \text{ ms}$).

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324 **Competing interests:**

325 The authors declare that no competing interests exist.

Figures

Figure 1. Experimental design for DXT measurement of TRPV1

(A) Side view of the sample chamber. N-terminally (His)₆-tagged TRPV1 was immobilized through a Ni-NTA (self-assembled monolayer formed on the polyimide films). Gold nanocrystals were attached at the extracellular side of TRPV1 via Met-tags introduced in the sequence. (B) Schematic of the DXT measurement. Pink-beam X-rays from the SPring-8 synchrotron radiation facility elicited trackable diffraction spots from the gold nanocrystals. The trajectories were projected and analyzed on the χ - θ coordinates, separately. The central three panels are representative motion of diffraction spot. The right panel shows piled data of Au (111) spots, representing trajectories. (C) Rotational angular displacement along θ and χ axes over time (4 ms). The 2,030 traces obtained from the ligand-free condition are presented. (D) Labeling positions of 2D diagram (upper panel) and 3D structures (lower panels) of TRPV1. The green arrows show Met-tags placed either at the outer pore (OP) region or voltage-sensor-like (VSL) domains of the extracellular side of TRPV1. The gold nanocrystals were specifically bound to the genetically introduced Met-tag (MGGMGM) in TRPV1 through Au-S binding. Highlighted residues in the 2D diagram are implicated in capsaicin-induced gating properties. (E) Mean square displacement (MSD) curves of TRPV1 motion for the χ axis. (F) Distributions of displacement over an interval time t ($\Delta t = 0.1$ ms) for the VSL-label. Histograms are fitted by the normal distribution (thick gray line), in which the mean μ and standard deviation σ .

345 **Figure 2. Velocity-based classification**

346 (A-C) Trajectory data. (A, B) Representative short-lived spots with 1.2- and 1.6-ms lifetime,
347 respectively. (C) Representative long-lived spots (blue arrows) with 3.6-ms lifetime. (D) Lifetime vs.
348 average step sizes over 0.1-ms time-interval. Presented data of OP-label with capsaicin. (E) The
349 MSD of OP-label with capsaicin. The diffusion constants were 9.87, 7.30 and 5.04 mrad^2/ms , for
350 short ($LT < 2.5$ ms), long ($2.5 \leq LT < 4$ ms), and the longest ($8 \leq LT < 10$ ms) lifetime groups.
351 respectively. (F, G) Representative probability density at $\Delta t = 1.5$ ms (left panels) and subtraction of
352 the probability density (right panels).

Figure 3. Mean plot of wild type TRPV1

(A) The mean plot and 95% confidence intervals for the angle χ at short lifetime group (LT < 2.5 ms, left) The rotation angle was mostly non-directed or slightly CW biased at both the OP-label (left) and VSL-label (right). (B) The mean plot at long lifetime group (2.5 ≤ LT < 4 ms) of OP-label (left) and VSL-label (right). Dark-filled periods of the top bars show the lifetime to which the data belong. (C) The distribution of angular displacement in 1 ms time interval. Data are displayed for capsaicin. Maximum and minimum values of angular displacement are shown at lower right (CCW bias) and left (CW bias) of each plot, respectively.

Figure 4. The rotational motion of the slowest-moving group.

The rotational motion of ligand-free and capsaicin-bound TRPV1 in the longest lifetime-filtering ($8 \leq LT < 10$ ms). Capsaicin showed CW, while ligand-free showed CCW biased at both the OP-label (left) and VSL-label (right). The mean plots (circles) and 95% confidence intervals (bars) of the 8-10 ms lifetime group were presented. The number of trajectories used for the analysis were: for 371 (OP of ligand-free), 296 (OP of capsaicin), 353 (VSL of ligand-free) and 177 (OP of capsaicin).

367 **Figure 5. Mean plot of the Y511A mutant and structural comparison of closed and open form**
368 **of TRPV1**

369 **(A)** The mean plots (circles) and 95% confidence intervals (bars) for the angle χ of the long lifetime
370 filtering ($2.5 \leq LT < 4$ ms) for the OP-label (left panels) and VSL-label (right panels). Upper panels
371 demonstrate the non-biased motion of Y511A at the ligand-free state (open circles). Lower panels
372 show the capsaicin-induced biased motion of Y511A toward positive (CCW) direction (red open
373 circles). **(B)** Superimposition of apo-TRPV1 (PDB: 3J5P, grey) and DkTx/RTX-TRPV1 (3J5Q, blue).
374 The ankyrin repeat domains were held as a fulcrum. Rotational angles after DkTx/RTX binding was
375 calculated at the glycine 602 (OP-label) and the tyrosine 463(VSL-label). Both the OP- and VSL-
376 positions rotated clockwise direction, resulting in 10.6 and 7.1 degrees, respectively.

Supporting information

Figure S1 | Expression and function of TRPV1 receptors used in this experiment.

(A) Expression of VSL-label TRPV1, OP-label TRPV1, VSL-label Y511A mutant, and OP-label Y511A mutant on the HEK293 cells were confirmed by the Western blotting of the anti-FLAG antibodies. The right-end lane is a control transfection by the vector only. The arrowhead shows the position of TRPV1 receptors. (B) Responses to the capsaicin were tested by the calcium influx assay using the FlexStation 3 system with the FLIPR Calcium 6 reagent (Molecular Devices Inc.). Mean values of 2-4 independent measurements were plotted. EC_{50} was $0.088 \mu M$ (FLAG-TRPV1), $1.32 \mu M$ (VSL-label TRPV1) and $0.245 \mu M$ (OP-label TRPV1), respectively. (C) Gel-filtration profiles (Superdex 200 column) of TRPV1 purification. Buffer composition was; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM $MgCl_2$, 1 mM n-Decyl- β -D-maltoside).

389 **Figure S2 | DXT measurement system (upper panels) and the X-ray flux in PIN diode detector**
 390 **for the incident beam (lower panel)** X-rays from the beamline (BL40XU, SPring-8, Japan) with
 391 energy widths from 14.0-16.5 keV (undulator gap = 30.1 mm) were used for DXT measurements.

392 **Figure S3 | The VHC provide symmetric distribution for OP-label.**

393 Before classification of lifetime, the distribution were agreed well by Gaussian function at short time
 394 intervals (= 0.1 ms) to large time intervals (= 7.5 ms).

Figure S4 | Subtraction in the probability density between the positive value and the negative value for OP-label at $\Delta t = 0.1$ ms.

(A) Subtraction of the probability density for all detected trajectories group ($0.4 \leq \text{lifetime (LT)} < 10$ ms) with capsaicin. (B) Subtraction of the probability density for long ($2.5 \leq \text{LT} < 4$ ms) with capsaicin. Comparing with long LT group which shows significant negative difference, all detected trajectories group didn't show any obvious rotational bias.

401 **Figure S5 | Mean-plot of the VSL-label for the further classified short (the lifetime (LT) < 2.5**
402 **ms) lifetime group.**
403 Different length of lifetime filtering was further applied to the short lifetime group (LT < 2.5 ms),
404 and the mean-plots of subgroups were calculated. Rotational motion of the subgroups was also
405 confirmed to be non-biased as shown in the result from the short lifetime group (Fig. 3A). Applied
406 lifetime filtering is shown at upper left of each panel.

407 **Figure S6 | The mean plots of the WT and Y511A against capsaisin for the short lifetime group**
 408 **(LT < 2.0 ms).**

409 Like the motion observed in the WT (Fig. 3A), Y511A in the short lifetime groups also did not show
 410 any specific rotational bias. Dark-filled area of the top right bar shows the applied lifetime periods.

Figure S7 | Superimposed views of closed and open-form of TRPV1.

(A) The ankyrin repeats domain at residues 111-143 was held as a fulcrum, and the structures were viewed and compared from the extracellular side. Apo (PDB: 3J5P, gray) and capsaicin-bound partially open (3J5R, magenta) structures were superimposed. (B) Apo- (PDB: 3J5P, grey) and DkTx/RTX-bound full-open (3J5Q, blue) structures were superimposed (reposted from the Fig. 5B). No significant conformational change was shown both at the OP- and VSL- positions by capsaicin, while the DkTx/RTX, which induces full-open structure, generated the CW rotation biases. Positions of OP (Gly 602)- and VSL (Tyr 463)- labels were indicated by circles.

VSL-label

419

	Ligand-free (10,465)	Capsaicin (8,026)	AMG9810 (10,578)
D_x (mrad^2/ms)	4.03	5.88	5.68
α	1.37	1.51	1.38
σ^2 (mrad^2)	0.59	0.86	0.53

OP-label

420

	Ligand-free (12,108)	Capsaicin (10,660)	AMG9810 (11,090)
D_x (mrad^2/ms)	4.30	4.39	4.61
α	1.49	1.57	1.68
σ^2 (mrad^2)	0.75	0.83	0.85

421 **Table S1| Parameter of MSD for wild type of TRPV1**

422 The MSD curve were fitted by the following function: $\delta^2(t) = D_\alpha t^\alpha + 2\beta^2$. D_α is the anomalous
423 diffusion constant, a non-linear relationship to time, α called subdiffusion ($1 > \alpha > 0$) or
424 superdiffusion ($\alpha > 1$) and β called measurement error.

	short	middle	long
	LF:0.4-2.5 ms	LF:2.6-4.0 ms	LF:8.0-10 ms
D_x (mrad^2/ms)	9.87	7.30	5.04
α	1.27	1.40	1.39
σ^2 (mrad^2)	0.59	0.46	0.29

425 **Table S2| Parameter of MSD for each Lifetime group in OP-label with capsaicin**

426 The MSD curve were fitted by the following function: $\delta^2(t) = D_\alpha t^\alpha + 2\beta^2$. D_α is the anomalous
427 diffusion constant, a non-linear relationship to time, α called subdiffusion ($1 > \alpha > 0$) or
428 superdiffusion ($\alpha > 1$) and β called measurement error.