Cell Reports, Volume *28*

Supplemental Information

Development of a Mouse Pain Scale Using Sub-second Behavioral Mapping and Statistical Modeling

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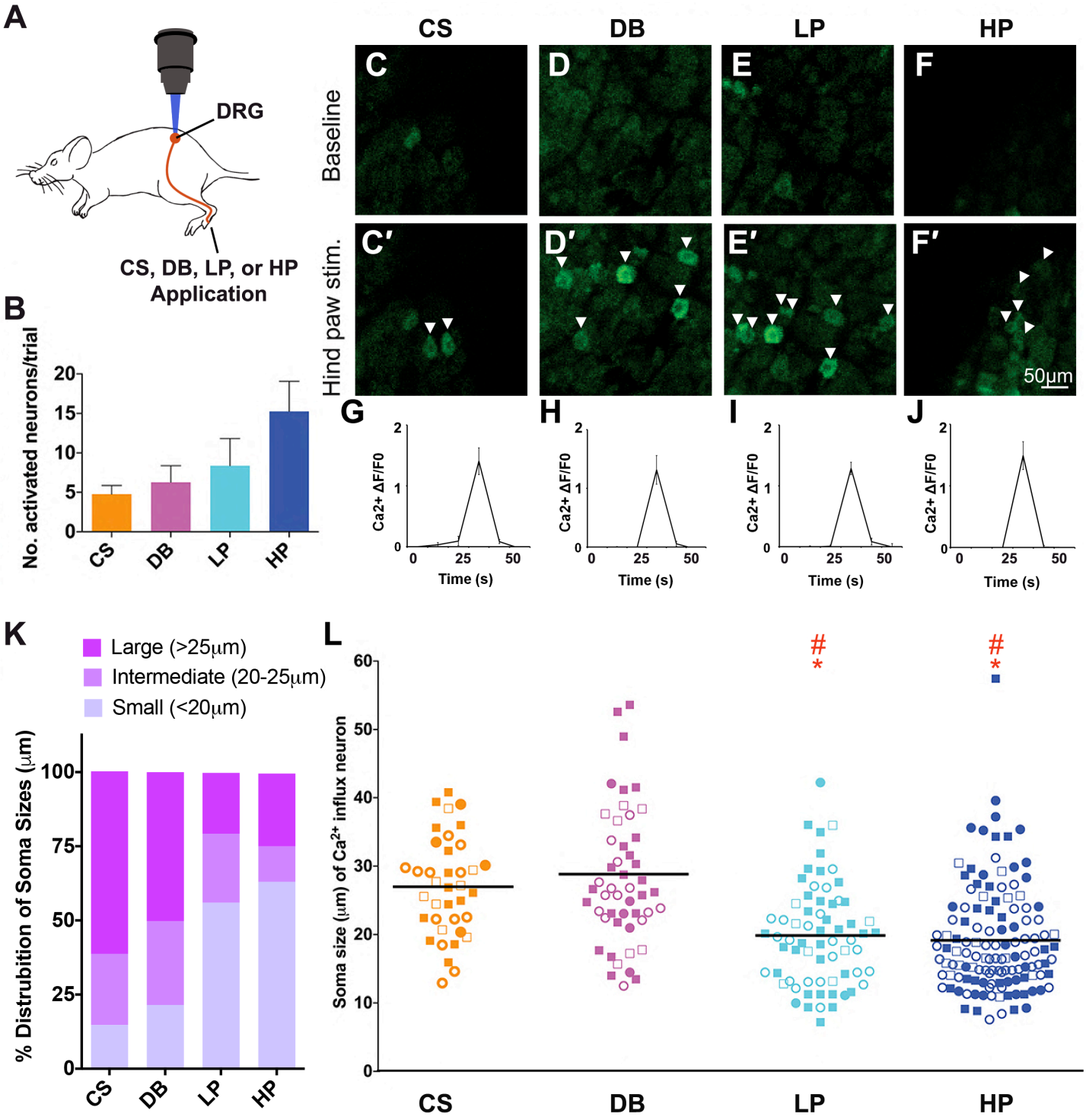
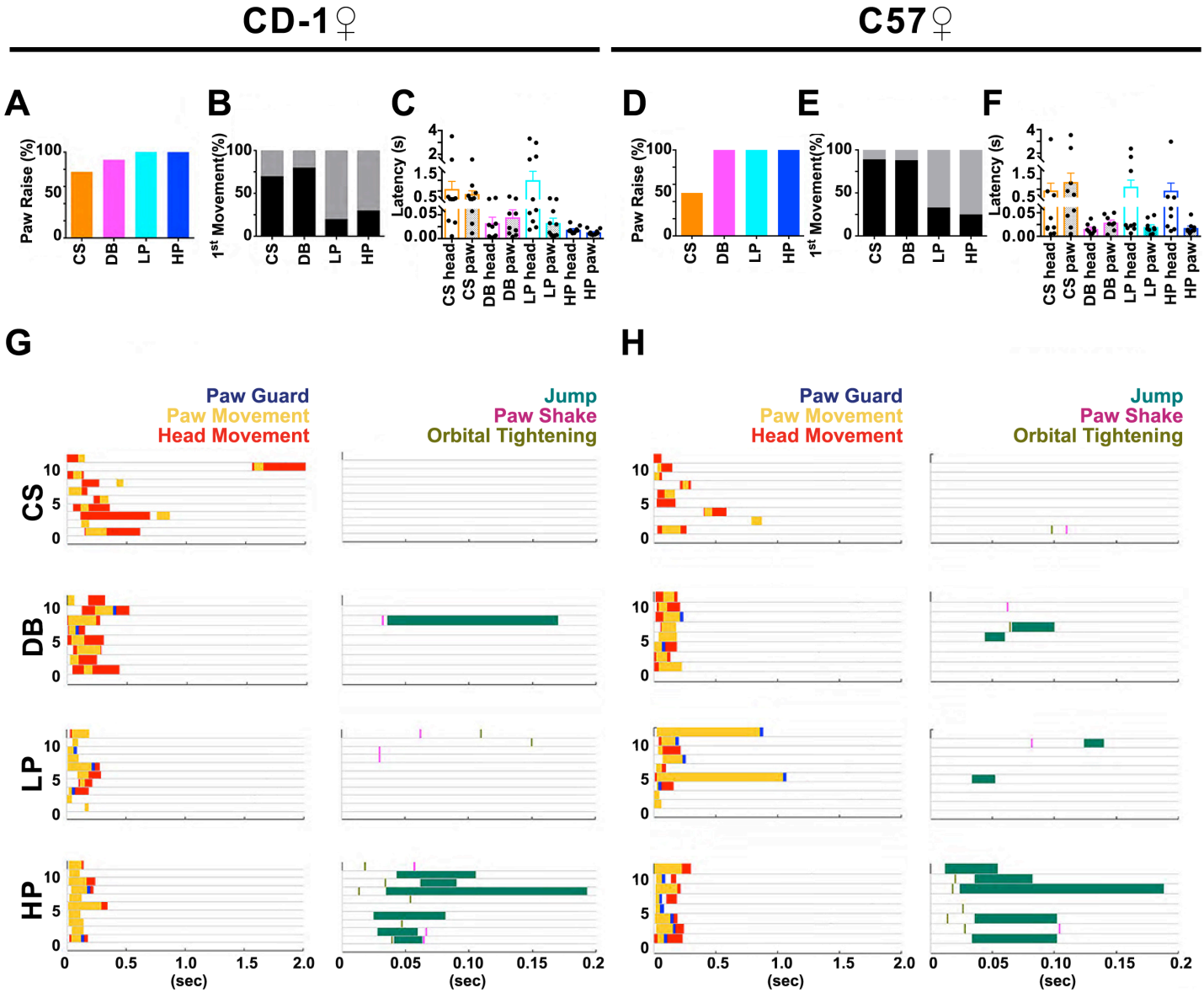
Suplemental Figure 1



Supplemental Figure 1 (related to Figure 1). Whole animal DRG neuronal calcium imaging combined with hind paw stimulation of natural mechanical stimuli. (A) Schematic of *Pirt-GCAMP6* anesthetized animal undergoing dorsal root ganglion (DRG) calcium imaging while stimulating the hind paw with cotton swab (CS), dynamic brush (DB), light pinprick (LP), or heavy pinprick (HP). (B) Number of activated neurons per DRG, determined by Ca^{2+} influx ($\Delta F/F_0 > 20\%$), in *Pirt-GCAMP6* mice (2 trials/mouse, n = 4). (C-F) Images of *GCAMP6* florescence before (baseline) and during hind paw stimulation. Scale bar: 50 μm for all images. (G-J) Ca^{2+} transients averaged together from all mice with each stimulus (abbreviated stimulus name is above C-F) showing time-windows of activating neurons before, during, and after stimulus application. Note: stimulus was applied for approximately 1 s at the 24 s timepoint. Error bars represent SEM. (K) Soma size per activated neuron, determined by Ca^{2+} signal, in *Pirt-GCAMP6* mice. For each animal tested, percentage of small, medium, or large diameter neurons are plotted. Data are plotted according to the stimuli used in panels C-J. (L) Graph shows raw values combined for all animals given four distinct stimuli with each animal distinguished as either open circles, closed circles, open squares, or closed squares. Each shape represents a single neuron. Red asterisks represent $p < 0.05$ when comparing CS to LP or CS to HP (LP or HP > CS), while red stars represent $p < 0.05$ when comparing DB to LP or DB to HP (LP or HP > CS).

Supplemental Figure 2



Supplemental Figure 2 (related to Figure 1). Sub-second temporal mapping of female mouse behavioral features in response to paw application of natural mechanical stimuli. (A) Percentage of paw raise towards a given stimuli for CD1 females. (B) First movement, whether head (black) or paw (grey), after stimulus application. (C) Latency of head and paw movement upon each stimulation. (D) Percentage of paw raise towards a given stimuli for C57 females. (E) First movement, whether head (black) or paw (grey), after stimulus application. (F) Latency of head and paw movement upon each stimulation. (G,H) Responses of CD1 and C57 female mice to paw stimulation of cotton swab (CS), dynamic brush (DB), light pinprick (LP), and heavy pinprick (HP) are plotted as raster plots, showing when six behavior features (color-coded in the figure) occurred after stimulus onset within the first 2 s or the first 200 ms. For each raster plot, the times when the behaviors occurred are shown on the X-axis, while the Y-axis and each horizontal line show a single trial/animal. (A, B, C, G) are data from CD1 female mice, and (D, E, F, H) are data from C57 female mice (n = 10 for CD1 and n = 8-9 for C57).

Supplemental Figure 3

A

Correlation Matrix

Parameters	Total Paw Time	Paw Air Time	Paw from Apex	Paw at Apex	Height	Velocity	Response Time	Head Turn Duration	Full Turn Duration	Total Time	Pain Score
Total Paw Time	1.000	1.000	0.998	0.897	0.151	0.008	-0.077	-0.122	0.000	0.230	0.116
Paw Air Time	1.000	1.000	0.999	0.895	0.147	0.011	-0.080	-0.122	0.002	0.233	0.117
Paw from Apex	0.998	0.999	1.000	0.902	0.141	0.011	-0.077	-0.118	0.006	0.233	0.116
Paw at Apex	0.897	0.895	0.902	1.000	0.215	0.095	-0.079	-0.083	0.017	0.196	0.162
Height	0.151	0.147	0.141	0.215	1.000	0.708	-0.239	-0.232	-0.237	-0.293	0.561
Velocity	0.008	0.011	0.011	0.095	0.708	1.000	-0.201	-0.250	-0.274	-0.324	0.498
Response Time	-0.077	-0.080	-0.077	-0.079	-0.239	-0.201	1.000	0.210	0.016	0.134	-0.201
Head Turn Duration	-0.122	-0.122	-0.118	-0.083	-0.232	-0.250	0.210	1.000	0.020	0.038	-0.237
Full Turn Duration	0.000	0.002	0.006	0.017	-0.237	-0.274	0.016	0.020	1.000	0.873	-0.148
Total Time	0.230	0.233	0.233	0.196	-0.293	-0.324	0.134	0.038	0.873	1.000	-0.213
Pain Score	0.116	0.117	0.116	0.162	0.561	0.498	-0.201	-0.237	-0.148	-0.213	1.000

B

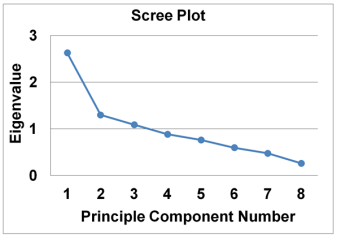
Iteration 1

Total Variance Explained

Principle Component	Initial Eigenvalues Total	% of Variance	Cumulative %
1	2.630	32.879	32.879
2	1.296	16.203	49.083
3	1.089	13.616	62.698
4	0.884	11.048	73.747
5	0.764	9.552	83.299
6	0.597	7.461	90.760
7	0.478	5.971	96.730
8	0.262	3.270	100.000

Component Matrix

Parameters	Principle Component 1	2	3
Height	0.866		
Velocity	0.804		
Pain Score	0.806		
Response Time	-0.374		-0.400
Paw Air Time		0.824	
Full Turn Duration		0.749	
Total Time			0.813
Head Turn Duration	-0.377		-0.558



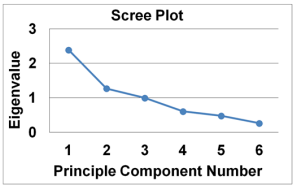
Iteration 2

Total Variance Explained

Principle Component	Initial Eigenvalues Total	% of Variance	Cumulative %
1	2.390	39.832	39.832
2	1.266	21.104	60.936
3	0.997	16.622	77.559
4	0.605	10.082	87.641
5	0.478	7.966	95.607
6	0.264	4.393	100.000

Component Matrix

Parameters	Principle Component 1	2
Height	0.895	
Velocity	0.858	
Pain Score	0.802	
Paw Air Time		0.830
Full Turn Duration	-0.397	0.691
Total Time		-0.329



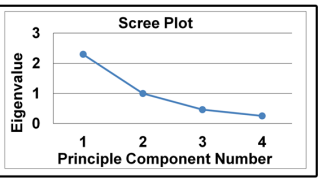
Iteration 3

Total Variance Explained

Principle Component	Initial Eigenvalues Total	% of Variance	Cumulative %
1	2.293	57.321	57.321
2	0.996	24.888	82.208
3	0.459	11.472	93.680
4	0.253	6.320	100.000

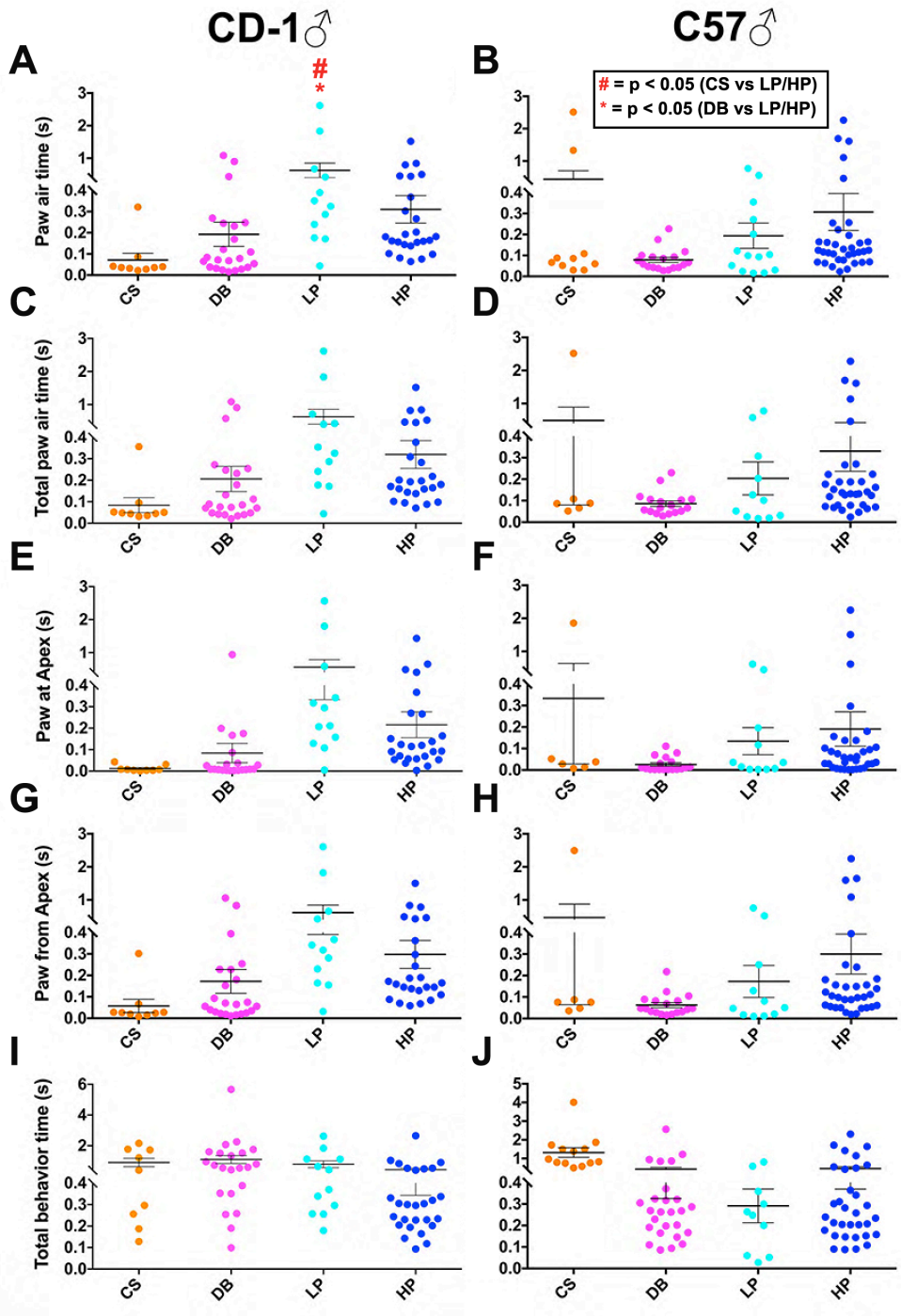
Component Matrix

Parameters	Component 1
Height	0.904
Velocity	0.873
Pain Score	0.819
Paw Air Time	0.205



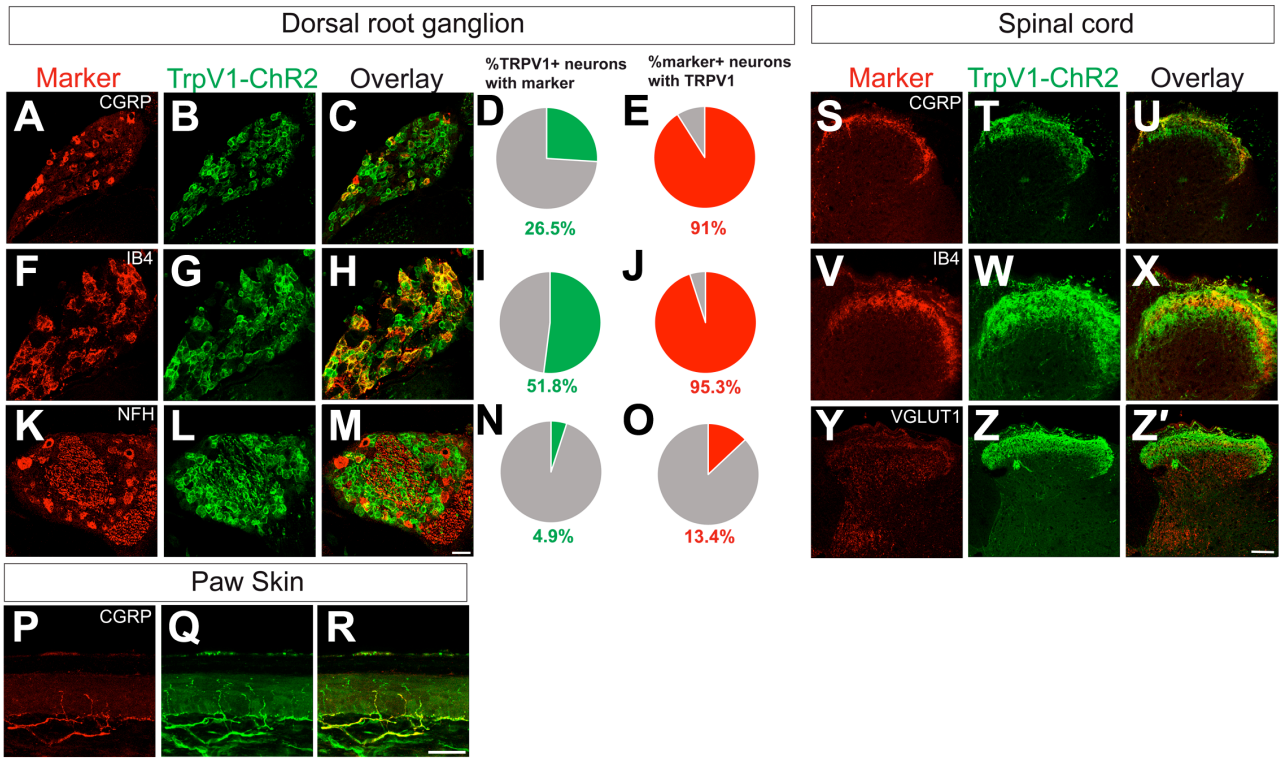
Supplemental Figure 3 (related to Figure 2). Exploratory Factor Analysis reveals four parameters that account for majority of variance. Data is derived from ~50% of trials from both C57 and CD1 males. (A) Correlation matrix between all 11 movement parameters. Cells marked by a gradient of red indicate correlations above 0.75. (B) Iterative exploratory factor analysis. Each iteration has three panels: 1) Cumulative "Total Variance Explained" for each principle component. Principle components with eigenvalue greater than 1.0 are highlighted blue. 2) "Component Matrix" with factor loadings for each parameter that makes up the associated principle component highlighted blue. 3) Scree plot for each principle component. Note that each iteration removes parameters that either have a low factor loading (< 0.35) or cross-load onto multiple factors (highlighted red).

Supplemental Figure 4



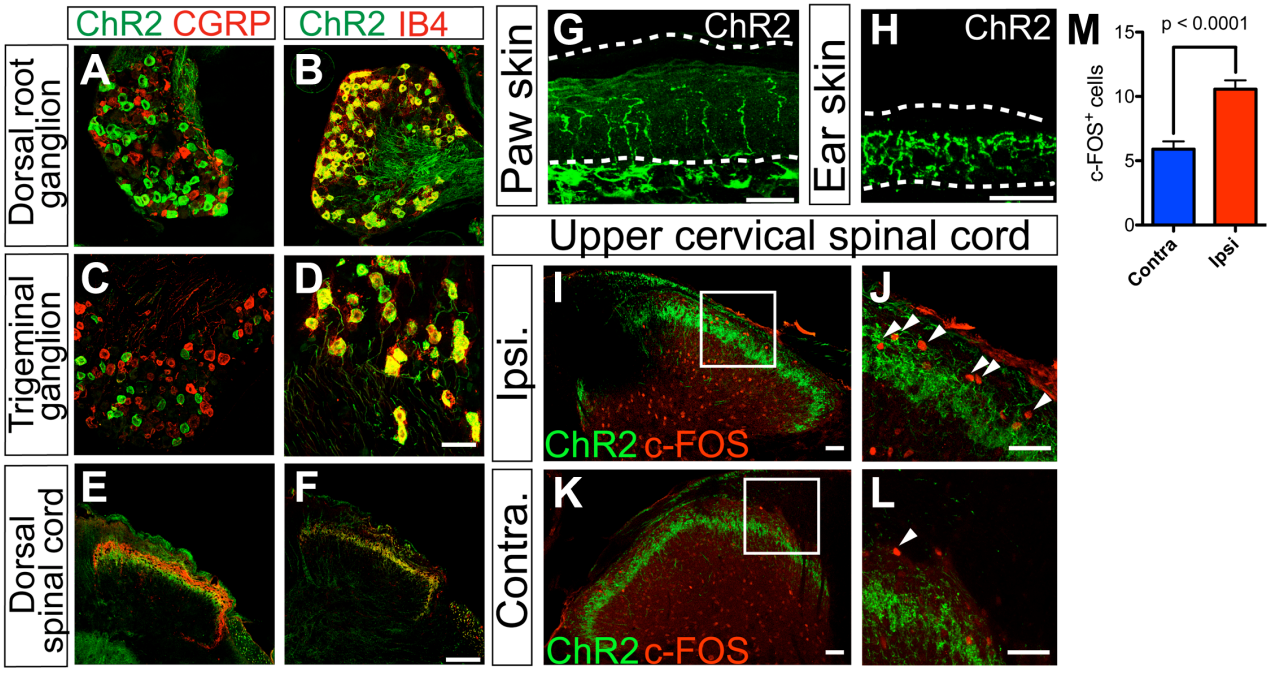
Supplemental Figure 4 (related to Figure 2). Stimulus-evoked movement features excluded from further analyses with PCA and SVM. (A, B) Paw air time measurements for CD1 males (A) and C57 males (B) after application of the four natural stimuli mentioned in the main text. Paw air time refers to the time when the animal's stimulated hind paw is in the air. (C, D) Total paw air time measurements for CD1 males (C) and C57 males (D) after application of the four natural stimuli mentioned in the main text. Total paw air time refers to the time when the animal's stimulated hind paw is in the air, including the time when it first moves in paw before lifting away from the surface. (E, F) Paw at apex measurements for CD1 males (E) and C57 males (F) after application of the four natural stimuli mentioned in the main text. Paw at apex refers to the time when the animal's stimulated hind paw is held at its maximal point in the air. (G, H) Paw from apex measurements for CD1 males (G) and C57 males (H) after application of the four natural stimuli mentioned in the main text. Paw from apex refers to the time when the animal's stimulated hind paw is coming down from its maximal height towards placement back on the surface. (I, J) Total behavior time measurements for CD1 males (I) and C57 males (J) after application of the four natural stimuli mentioned in the main text. Total behavior time refers to the time the animal first begins a movement (either head turn or paw lift) until the time these movements are completed (n = 10 for all groups).

Supplemental Figure 5



Supplemental Figure 5 (related to Figure 6). Histology of the dorsal root ganglia, spinal cord, and plantar paw skin of *Trpv1^{Cre}; Ai32* mice. (A-C) DRG sections immunostained with antisera directed against GFP, which recognize ChR2-EFYP fusion protein, and CGRP, and the percentages of overlap are shown with bar graphs (D, E). Corresponding double immunostaining was done on spinal cord tissue (S-U). (F-H) DRG sections immunostained with IB4 and antiserum directed against GFP, and percentages of overlap are shown with bar graphs (I, J). Corresponding double immunostaining was done on spinal cord tissue (V-X). (K-M) DRG sections immunostained with antisera directed against GFP and NFH and percentages of overlap are shown with bar graphs (N, O). Corresponding double immunostaining was done on spinal cord tissue with antisera against GFP and VGLUT1 (Y-Z'). Plantar paw skin double immunostaining was performed with antisera directed against GFP and NFH (P-R). n = 3 mice between P21-P28 for histology with DRG, spinal cord, and plantar paw skin. Scale bars: 50 μm.

Supplemental Figure 6



Supplementary Figure 6 (related to Figure 6). Genetic targeting and activation of ChR2 in MRGPRD$^+$ neurons. (A, B) Representative confocal image of immunostaining with dorsal root ganglia sections of *Mrgprd-ChR2* mice, showing no overlap between DRG neurons expressing ChR2-EYFP and CGRP, but complete overlap between those expressing ChR2-EYFP and binding IB4. (C, D) Similar expression patterns are observed after immunostaining with the trigeminal ganglia sections for antisera that detect ChR2-EYFP, CGRP, and IB4. (E, F) Immunostaining with the dorsal spinal cord sections of *Mrgprd-ChR2* mice showing efficient targeting of ChR2-EYFP to central terminals that do not overlap with CGRP+ (E) but IB4+ (F) central terminals. (G, H) Immunostaining showing efficient targeting of ChR2-EYFP to peripheral terminals in the dermal plantar paw (G) and ear skin (H) of *Mrgprd-ChR2* mice. (I-L) Immunostaining of c-FOS with the upper cervical spinal cord sections following optogenetic ear stimulation of *Mrgprd-ChR2* mice shows increased number of c-FOS$^+$ neurons in the ipsilateral (blue light) superficial dorsal horn (I, J) compared to the contralateral side (no light) (K, L). J, L are magnified from the white box areas in I, K. (M) Quantification of c-FOS cells following optogenetic stimulation of *Mrgprd-ChR2* mice. P-value is from student's t-test and error bars represent SEM. n = 3 mice. Scale bars: 50 μm.