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4 **Rabies anterograde monosynaptic tracing reveals organization of spinal sensory circuits**

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24 ***Abstract***

25           Somatosensory neurons detect vital information about the environment and internal  
26 status of the body, such as temperature, touch, itch and proprioception. The circuit  
27 mechanisms controlling the coding of somatosensory information and the generation of  
28 appropriate behavioral responses are not clear yet. In order to address this issue, it is  
29 important to define the precise connectivity patterns between primary sensory afferents  
30 dedicated to the detection of different stimuli and recipient neurons in the central nervous  
31 system. In this study we used a rabies tracing approach for mapping spinal circuits receiving  
32 sensory input from distinct, genetically defined, modalities. We analyzed the anatomical  
33 organization of spinal circuits involved in coding of thermal and mechanical stimuli and  
34 showed that somatosensory information from distinct modalities is relayed to partially  
35 overlapping ensembles of interneurons displaying stereotyped laminar organization, thus  
36 highlighting the importance of positional features and population coding for the processing  
37 and integration of somatosensory information.

## 38 ***Introduction***

39           The somatosensory system is responsible for detecting a wide variety of sensory  
40 information and generate appropriate behavioral responses. The circuit mechanisms  
41 controlling the detection of different modalities and its transformation into motor actions are  
42 not completely understood. Much progress has been made in the characterization of primary  
43 somatosensory neurons in the peripheral nervous system, and physiological and molecular  
44 descriptions of different subtypes specialized in the detection of discrete modalities exist  
45 (Abraira and Ginty, 2013; Vriens et al., 2014; Le Pichon and Chesler, 2014; Zampieri and De  
46 Nooij, 2020). However, comparatively little is known about the logic underlying the coding  
47 of sensory information and the generation of appropriate motor behaviors.

48           The specialization of peripheral afferents for the detection of distinct stimuli  
49 represents the foundation underlying the specificity theory, which proposes that different  
50 sensory information is encoded along parallel dedicated pathways or labelled lines (Norrzell  
51 et al, 1999). An alternative view, supported by studies on pain, is based on pattern theory and  
52 it postulates that perception is generated by temporal summation of various peripheral inputs  
53 at the level of relay centers in the central nervous system (CNS; Perl, 2007). More recently, a  
54 synergistic model, population coding, has been proposed (Ma, 2010). It suggests that cross-  
55 talk between labelled lines in the CNS is responsible for coding of sensory perception. This  
56 hypothesis highlights the functional specialization of primary sensory afferents and postulate  
57 the existence of specific patterns of connectivity with second order neurons in the spinal cord  
58 and medulla. Thus, defining the location and identity of spinal interneurons receiving input  
59 from distinct sensory modalities represent an important step toward resolving the circuit  
60 mechanisms controlling the coding of somatic sensation. However, systematic analysis has  
61 been so far precluded by the lack of high-throughput approaches that directly links the  
62 subtype identity of primary afferents with their spinal targets (Bokiniec et al., 2018).

63 Transsynaptic tracing using rabies virus is a powerful tool for mapping neural circuits in the  
64 brain and spinal cord (Wickersham et al., 2007; Callaway and Luo, 2015). Rabies virus has  
65 also been shown to infect primary sensory neurons in the peripheral nervous system and  
66 move in the anterograde direction to spread into synaptic targets into the CNS (Ugolini, 2010;  
67 Velandia-Romero et al., 2013; Bauer et al., 2014). However, several limitations have  
68 hampered the use of rabies tracing for systematic analysis of spinal sensory circuits. First, not  
69 all sensory afferents are susceptible to rabies virus infection (Albisetti et al., 2017). Second,  
70 neuronal infection after peripheral, intramuscular or cutaneous, rabies injection is only  
71 efficient in neonatal mice (Stepien et al., 2010; Zampieri et al., 2014; Zhang et al., 2015).  
72 Finally, questions have been raised about the directionality of the transfer and whether it  
73 represents bona fide anterograde tracing of postsynaptic targets or retrograde labeling of  
74 presynaptic neurons providing axo-axonic input to sensory afferents (Zhang et al., 2015).

75 In this study, we show that intraspinal injection of EnvA-pseudotyped rabies virus can  
76 retrogradely infect TVA expressing neurons in the dorsal root ganglia (DRG) without any  
77 obvious limitation in either tropism or timing, and jump in the anterograde direction into  
78 second order spinal neurons allowing high resolution mapping of post-sensory circuits.  
79 Analysis of proprioceptive circuits resulted in identification of motor neurons and interneuron  
80 subtypes, that are well known post-synaptic partners, thus indicating genuine anterograde  
81 transsynaptic transfer. We applied this approach to identify spinal targets of sensory afferents  
82 detecting thermal and mechanical stimuli and observed a high degree of spatial segregation  
83 along dorso-ventral axis of the spinal cord. However, we identified convergence of distinct  
84 post-sensory circuits in mutual exclusive areas in dorsal laminae I-III, thus indicating  
85 important roles as hubs for integration of multiple sensory modalities. These findings  
86 highlight the functional relevance of the laminar organization of the spinal cord and

- 87 emphasize the essential role of positional features, as a determinant in the assembly and
- 88 function of sensory-motor circuits.

89 **Results**

90 **Retrograde infection of primary somatosensory neurons and anterograde transfer into**  
91 **second order neurons**

92 In order to characterize the anatomical organization of spinal circuits according to the  
93 somatosensory input they receive, we combined mouse genetic and rabies virus (RV, SAD  
94 B19 strain; Wickersham et al., 2007) monosynaptic tracing techniques (Figure 1A). To  
95 achieve cellular specificity in RV infection and subsequent monosynaptic transfer we used a  
96 mouse line driving conditional expression of the TVA receptor, the RV glycoprotein (G) and  
97 a nuclear GFP reporter in combination with Cre lines targeting defined subsets of  
98 somatosensory neurons (*Rosa26<sup>Lox-stop-LoxHTB</sup>* or *HTB*; Li et al., 2013). Expression of the TVA  
99 receptor and G are required for selective infection by EnvA pseudotyped G-deleted RV  
100 (RVΔG-mCherry/EnvA) and subsequent monosynaptic spreading, while the nuclear GFP  
101 reporter allows identification of starter cells (Figure 1A). We first focused on proprioceptive  
102 circuits that have been quite extensively characterized at anatomical and physiological levels  
103 (Zampieri et al., 2014; Balaskas et al., 2020). Thus, we crossed *parvalbumin::Cre (PV::Cre)*,  
104 which is expressed in proprioceptive sensory neurons and a subset of low-threshold  
105 mechanoreceptors (LTMR) with the *HTB* mouse line (Hippenmeyer et al., 2007; de Nooij et  
106 al., 2013). First, we confirmed the expression specificity of the *HTB* allele and found that at  
107 lumbar levels about 96% of GFP<sup>+</sup> DRG neurons were also PV<sup>+</sup> (Figure 1B and C). In  
108 addition, GFP was not detected in the lumbar spinal cord up to postnatal (p) day 10,  
109 indicating that neither the TVA receptor nor G are expressed in spinal neurons up to this  
110 stage (Figure S1A). In order to target sensory neurons independently of their subtype identity  
111 and peripheral target connectivity we delivered RV directly in the spinal cord to gain access  
112 to somatosensory afferents (Figure 1A). Unilateral stereotactic injection of RVΔG-  
113 mCherry/EnvA at lumbar (L) level 1 of p9 *PV::Cre<sup>+/-</sup>; HTB<sup>ff</sup>* (hereafter referred to as *PV<sup>HTB</sup>*)

114 mice resulted in the infection of PV<sup>+</sup> neurons in L2-L4 DRG (Figures 1A, D, E, F, and S1B).  
115 Next, we examined the spinal cord seven days after rabies injection and observed labeling of  
116 interneurons and motor neurons (Figure 1G and Video S1). In contrast, when we injected  
117 *PV::Cre<sup>+/-</sup>; HTB<sup>f/+</sup>* mice, we obtained infection of PV<sup>+</sup> DRG neurons but negligible labeling  
118 of spinal neurons (Figure S1C). This observation indicates that one copy of the *HTB* allele  
119 can promote sufficient expression of TVA to drive interaction with EnvA pseudotyped RV  
120 but not enough G to support transsynaptic transfer. Strikingly, we observed that the majority  
121 of neurons labeled in the spinal cord were located along or nearby the axonal trajectories of  
122 proprioceptive sensory afferents (Figures 1G and 1H). Altogether these data indicate that  
123 spinal injection of rabies results in retrograde infection of proprioceptive neurons and  
124 anterograde transsynaptic spreading into neuronal targets in the spinal cord.

125

## 126 **Organization and identity of second order neurons receiving proprioceptive input**

127 We next sought to characterize the spinal neurons labeled by rabies tracing (Figure  
128 2A). We generated three-dimensional maps of infected neurons and analyzed their positional  
129 organization in the spinal cord. The vast majority of second order neurons were located  
130 ipsilateral to the point of injection (Figures 2B, 2C and S2). Distribution along the dorso-  
131 ventral axis presented three distinct peaks corresponding to the dorsal, intermediate and  
132 ventral spinal cord (Figures 2B and 2D). Consistent with unbiased access to sensory afferents  
133 independent of their peripheral target, we found rabies-labelled motor neurons of both lateral  
134 and medial motor column identity in the ventral ipsilateral side (Figures 2A, 2C and S2). The  
135 connectivity patterns obtained from different tracing experiments were qualitatively and  
136 quantitatively reproducible as shown in individual maps, distribution and correlation analyses  
137 (“IN vs IN”  $r \geq 0.9$ ; “MN vs MN”  $r \geq 0.8$ ; Figures 2D, 2H and S2). We observed some  
138 variability in the amount of neuronal labelling in different experiments, however the ratio

139 between the number of starter cells and second order neurons, defined as the “connectivity  
140 index”, remained constant indicating that, under these conditions, rabies can reproducibly  
141 label ~5 spinal neurons for each primary sensory neuron infected (Figures 2E-G and Table  
142 S1). Interestingly, a similar level of transsynaptic transfer was previously reported in  
143 retrograde tracing experiments from motor neurons using the SAD B19 strain (Reardon et al.,  
144 2016). Next, we investigated the identity of spinal neurons labelled by rabies virus. Aside  
145 from motor neurons, several cardinal classes of spinal neurons are known to receive direct  
146 proprioceptive input (Eccles et al., 1957; Côté et al., 2018). We analysed the expression of  
147 markers that, along with positional information, define V2a, V1, V0 and dI4 identities at  
148 early postnatal stages (Zampieri et al., 2014; Bikoff et al., 2016). We found rabies labelled  
149 Chx10<sup>+</sup> V2a interneurons, FoxP2<sup>+</sup> V1 interneurons, ventrally positioned calbindin<sup>+</sup> Renshaw  
150 cells, and Lhx1<sup>+</sup> interneurons whose dorsal position is suggestive of dI4 identity (Figure S3).  
151 These findings confirm that rabies labels spinal neurons that are known to receive  
152 monosynaptic proprioceptive input and therefore represent genuine postsynaptic targets.

153

#### 154 **Anterograde tracing from thermosensitive neurons**

155 In order to explore the anatomical organization of spinal somatosensory circuits we  
156 mapped post-sensory neurons receiving input from different primary afferents. Since the  
157 *PV::Cre* line gives access to neurons of mechanoreceptive lineage, mainly proprioceptors and  
158 a subset of LTMR, we decided to focus on thermosensation, a distinct modality that is  
159 accessible using available mouse genetic tools. We took advantage of the *TRPV1::Cre* and  
160 *TRPM8::Cre* mouse lines that are known to target hot- and cold-sensing DRG neurons  
161 (Mishra et al., 2011; Yarmolinsky et al., 2016). *TRPV1* is transiently expressed during  
162 development by most sensory neurons of thermoceptive lineage, while *TRPM8* is restricted to  
163 a smaller subset of cold-sensing neurons (Dhaka et al., 2008; Mishra et al., 2011). Indeed,



164 GFP expression in *TRPV1::Cre<sup>+/-</sup>; HTB<sup>ff</sup>* and *TRPM8::Cre<sup>+/-</sup>; HTB<sup>ff</sup>* (hereafter referred to as  
165 *TRPV1<sup>HTB</sup>* and *TRPM8<sup>HTB</sup>*) mice revealed a clear difference in the labeling of DRG neurons  
166 (Figures 3A and B). This observation was confirmed by tracing sensory afferents, in  
167 *TRPV1::Cre<sup>+/-</sup>; Ai14<sup>ff/+</sup>* mice we found dense staining in the dorsal spinal cord while only  
168 sparse signal was detected in the case of *TRPM8::Cre<sup>+/-</sup>; Ai14<sup>ff/+</sup>* (Figures S4C-D). In both  
169 cases we did not detect any labeling of spinal neurons with either the *HTB* or the *Ai14*  
170 reporter lines (Figures 3E, 3F and S4C-D). As previously done for *PV<sup>HTB</sup>* experiments, we  
171 performed L1 unilateral stereotactic injection of RVΔG-mCherry/EnvA in p9 mice and  
172 performed analysis at p16. In both cases we obtained selective infection of GFP<sup>+</sup> DRG  
173 neurons (Figures 3A-C). We observed similar efficiencies in primary infection, however,  
174 because of the difference in the amount of sensory neurons expressing Cre in the *TRPV1::Cre*  
175 and *TRPM8::Cre* lines, the number of starter cells was much higher in *TRPV1<sup>HTB</sup>*  
176 experiments (Figure 3A, 3B, 3D, 3I and Table S1). Surprisingly, we did not observe a  
177 proportional increase in the number of second order neurons labeled in *TRPV1<sup>HTB</sup>* mice, thus  
178 resulting in a low connectivity index (Figures 3I-K and Table S1). Next, we examined the  
179 spinal cords of rabies injected *TRPV1<sup>HTB</sup>* and *TRPM8<sup>HTB</sup>* mice and found extensive labeling  
180 in the ipsilateral side with higher incidence of RV<sup>+</sup> neurons in the dorsal horn that sharply  
181 decreased in the intermediate and ventral spinal cord (Figures 3E-H). The overall spatial  
182 organization of RV<sup>+</sup> neurons in *TRPV1<sup>HTB</sup>* and *TRPM8<sup>HTB</sup>* experiments were qualitatively  
183 similar and injections reproducible, as shown by single maps, distribution, and correlation  
184 analyses (Figures S4A-B, S4E). These data show that rabies can be used to trace from distinct  
185 primary somatosensory neuron subtypes and indicate that spinal sensory circuits encoding for  
186 different modalities, such as thermosensation and proprioception, are kept mostly separated,  
187 highlighting the functional specialization of the dorsal and ventral spinal cord in the control  
188 of sensory processing and motor control.

189

## 190 **Organization of post-sensory circuits in the dorsal laminae of the spinal cord**

191         Recent studies demonstrated the importance of topographic organization of dorsal  
192 spinal interneurons for encoding reflexes mediated by inflammatory and noxious stimuli  
193 (Gatto et al., 2021; Peirs et al., 2021). Thus, we asked whether the distribution of neurons  
194 labelled in  $PV^{HTB}$ ,  $TRPVI^{HTB}$ , and  $TRPM8^{HTB}$  may reveal the anatomical basis for the  
195 functional specificity of spinal somatosensory circuits. In  $PV^{HTB}$  experiments we found 43%  
196 of rabies-labeled neurons in the intermediate spinal cord (defined as the dorso-ventral area  
197 from 0 to 300 $\mu$ m) and a similar number of cells in the ventral (23%; 0 to -600 $\mu$ m) and dorsal  
198 (29%; 300 to 600 $\mu$ m) areas (Figure 4A). In contrast, the majority of neurons traced after  
199 rabies injections in  $TRPVI^{HTB}$  and  $TRPM8^{HTB}$  mice were located in the dorsal spinal cord  
200 (Figure 4A;  $TRPVI^{HTB}$  = 78% and  $TRPM8^{HTB}$  = 65%). Correlation analysis confirmed this  
201 observation by showing that Cartesian coordinates of  $RV^+$  neurons in  $TRPVI^{HTB}$  and  
202  $TRPM8^{HTB}$  experiments highly correlate with each other but not with the ones from  $PV^{HTB}$   
203 (“ $TRPVI^{HTB}$  vs  $TRPM8^{HTB}$ ,”  $r \geq 0.85$ ; “ $TRPVI^{HTB}$  or  $TRPM8^{HTB}$  vs  $PV^{HTB}$ ,”  $r \leq 0.55$ ; Figure  
204 4B). Despite the broad dorso-ventral segregation in the distributions of neurons receiving  
205 thermal and mechanical information, an area of potential overlap is evident in the dorsal  
206 spinal cord (Figure 4A). We used staining for  $PKC\gamma$ , a marker for lamina IIi and III (Polgar et  
207 al., 1999), as an internal reference for assessing relative positioning of dorsal interneurons  
208 labelled in  $PV^{HTB}$ ,  $TRPVI^{HTB}$ , and  $TRPM8^{HTB}$  experiments (Figure 4C). The data indicate that  
209 in  $PV^{HTB}$  experiments  $RV^+$  neurons are rarely found above lamina IIi, as opposed to  
210  $TRPVI^{HTB}$  mice where  $RV^+$  neurons are located mostly in lamina I and IIo, largely  
211 overlapping with the CRGP termination zone (Figures 4C-E and G). In addition, in  $PV^{HTB}$   
212 experiments we observed a subset of neurons displaying prominent laminar positioning  
213 mostly overlapping with  $PKC\gamma$  labeling, an area known to receive extensive input from

214 cutaneous LTMR (Figures 4C, 4D and 4E; Abaira et al., 2017). In contrast, spinal neurons  
215 traced in *TRPM8<sup>HTB</sup>* mice presented a more homogenous distribution across dorsal layers,  
216 thus resulting in one area of partial overlap with *TRPV1<sup>HTB</sup>* traced neurons in laminae I-IIo  
217 and one of partial overlap with *PV<sup>HTB</sup>* traced neurons in laminae IIi-III (Figure 4F).  
218 Altogether, these data indicate that interneurons residing in the superficial laminae can be  
219 divided into at least three different populations according to the sensory input they receive.

220 ***Discussion***

221 In order to understand the functional organization of spinal circuits controlling the  
222 processing of sensory information, it is critical to determine the patterns of connectivity  
223 between distinct primary sensory neuron subtypes and their targets in the central nervous  
224 system. In this study, by combining mouse genetics and rabies monosynaptic tracing  
225 techniques we identified an approach to directly link sensory input from defined, modality  
226 specific, primary afferents to neuronal targets in the spinal cord and analyzed the anatomical  
227 organization of spinal circuits encoding thermal and mechanical information.

228 The approach takes advantage of the ability of primary sensory neurons to support  
229 rabies transsynaptic transfer in the anterograde direction (Ugolini, 2010; Velandia-Romero et  
230 al., 2013; Bauer et al., 2014; Zampieri et al., 2014). In contrast to previous studies that used  
231 peripheral rabies injection, either cutaneous or intramuscular, to infect sensory neurons  
232 through their terminals, we opted for stereotactic injection of EnvA pseudotyped rabies virus  
233 in the spinal cord to infect TVA-expressing neurons through their central afferents. This route  
234 has two main advantages. First, the efficiency of rabies infection of DRG neurons, via their  
235 peripheral terminals is known to decrease rapidly within the first neonatal days, essentially  
236 restricting the experimental window from p1-p4 (Zampieri et al., 2014; Zhang et al., 2015).  
237 This limitation does not apply to intraspinal injection, thus opening the way for studying the  
238 organization of spinal post-sensory circuits during and after postnatal development in  
239 physiological or disease models. Second, intraspinal injection allows unbiased access to all  
240 sensory afferents projecting at a desired spinal level independent of their identity or pattern of  
241 peripheral innervation (i.e.: hairy vs glabrous skin, cutaneous vs muscle, etc.), in principle  
242 allowing direct comparisons of post-sensory circuits from different modalities without any  
243 limitation.

244 We used a mouse genetic strategy to specify starter cells by driving conditional  
245 expression of the TVA receptor, G protein and a reporter under control of Cre recombinase.  
246 This is an effective and relatively simple method for driving transgene expression in all  
247 neurons of interest. However, it requires a high degree of specificity in the Cre line, otherwise  
248 transient or leaky expression could result in the generation of multiple sets of cells capable of  
249 supporting rabies infection and transsynaptic tracing. For this reason, we carefully analyzed  
250 the patterns of the nuclear GFP and tdTomato reporters in the DRG and spinal cord of the Cre  
251 lines employed in this study. In order to ensure more stringent specificity, intersectional  
252 genetic and viral strategies can be used. For example, complementation of TVA and G  
253 expression using peripheral AAV injection in combination with rabies intraspinal delivery  
254 could eliminate specificity issues common to many Cre lines.

255 In agreement with a previous report, we did not find any obvious restriction in the  
256 ability of EnvA-pseudotyped rabies virus to infect TVA expressing somatosensory neuron  
257 (Albisetti et al., 2017). However, in comparison to TRPM8 and PV experiments, we observed  
258 relatively low connectivity when tracing from *TRPV1<sup>HTB</sup>* mice. Our data do not allow to  
259 distinguish whether this observation reflects an intrinsic property of these circuits or could  
260 hint at a limited ability of a subset of *TRPV1::Cre* neurons to support rabies spreading. It has  
261 been suggested that neural activity may have an important role in promoting efficient rabies  
262 transsynaptic transfer. Many nociceptors are labelled by the *TRPV1::Cre* line that because of  
263 the controlled conditions of laboratory mouse housing may not be active, thus possibly  
264 limiting their contributions to rabies tracing. A similar scarcity in connectivity has been  
265 previously shown in tracing experiments from *TRPV1::Cre* sensory neurons after rabies  
266 cutaneous injection (Zhang et al., 2015). The authors interpreted their results as an indication  
267 that transsynaptic labeling from sensory neurons represents retrograde transfer into  
268 presynaptic neurons through relatively infrequent axo-axonic synapses instead of anterograde

269 transfer into postsynaptic targets (Zhang et al., 2015). Analysis of neuronal identity and  
270 position in  $PV^{HTB}$  experiments strongly support anterograde transfer into postsynaptic targets,  
271 as we consistently observe labeling of motor neurons and spinal interneurons that are well-  
272 known recipients of monosynaptic input from proprioceptive sensory afferents (Eccles et al.,  
273 1957; Zampieri et al., 2014; Bikoff et al., 2016; Côté et al., 2018).

274 In order to reveal the anatomical organization of spinal somatosensory circuits, we  
275 used three different mouse lines,  $PV::Cre$  to label proprioceptive neurons and a subset of  
276 LTMR,  $TRPV1::Cre$  to label thermosensitive neurons, and  $TRPM8::Cre$  to label cold-sensing  
277 neurons (Hippenmeyer et al., 2007; Mishra et al., 2011; Yarmolinsky et al., 2016). We were  
278 therefore able to map neurons involved in the detection of two different stimulus modalities,  
279 proprioception and thermosensation, as well as circuits for more defined sensory features,  
280 cutaneous mechanoreceptors and cold sensing neurons. Positional analysis of post-sensory  
281 neurons revealed shared and distinct features of spinal somatosensory circuits. First, in all  
282 cases analyzed, we observed a very prominent ipsilateral bias in connectivity, with very  
283 limited labeling of contralateral neurons, indicating that the first relay stations processing  
284 somatic sensation do not directly integrate information coming from both sides of the body.  
285 Second, post-sensory neurons receiving thermal and proprioceptive information are mostly  
286 segregated along the dorso-ventral axis highlighting the functional separation of the dorsal  
287 and ventral spinal cord for sensory processing and motor control, respectively. Third, at a  
288 finer level of resolution, the anatomical organization of post-sensory circuits in the dorsal  
289 horn reflects the recently described functional specialization of superficial spinal interneurons  
290 in laminae I-IIo for encoding reflexes mediated by inflammatory and noxious stimuli, and of  
291 deeper interneurons in laminae Iii-IV for sensory-motor behaviours driven by mechanical  
292 inputs (Gatto et al., 2021; Peirs et al., 2021). Interneurons labeled in  $TRPV1^{HTB}$  experiments,  
293 that include afferents detecting noxious thermal stimuli are present at higher density in

294 lamina I and IIo, neatly segregated from the ones traced in *PV<sup>HTB</sup>* experiments, representing  
295 inputs relaying proprioceptive and cutaneous mechanoreceptive information, that are found in  
296 deeper layers starting from lamina III. Interestingly, spinal targets of afferents traced in  
297 *TRPM8<sup>HTB</sup>* experiments, that detect non noxious thermal information, present a more  
298 homogenous distribution throughout the dorsal horn, selectively overlapping with TRPV1-  
299 labelled neurons in laminae I-III and PV output areas in laminae IIo-III, thus indicating that  
300 even somatosensory information coming from distinct modalities is not strictly kept separated  
301 at the level of first-order spinal neurons. Altogether, these findings support a population  
302 coding model where different, modality specific, sensory inputs converge on ensembles of  
303 spinal interneurons that present stereotyped spatial organization and control different sensory-  
304 motor functions (Gradwell and Abaira, 2021).

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## 315 **Material and Methods**

### 316 **Mouse strains**

317 Animals were housed in the facility with controlled environmental parameters under a  
318 12h light/ 12h dark cycle and fed with standard chow. The following strains of mice were  
319 used in this study: *PV::Cre* (Hippenmeyer et al., 2007), *TRPV1::Cre* (Mishra et al.,2011),  
320 *TRPM8::Cre* (Yarmolinsky et al., 2006), *Rosa-lsl-HTB* (Li et al.,2013) and *Rosa-lsl-*  
321 *tdTomato* (Ai14, Jackson Laboratory). All animal experiments were approved by the  
322 Regional Office of Health and Social Affair Berlin (LAGeSo) and performed in compliance  
323 with the German Animal Welfare Act.

324

### 325 **Production of pseudotyped glycoprotein deficient rabies virus**

326 RVΔG-mCherry/EnvA was produced with minor modifications as previously  
327 described (Wickersham et al., 2010). BHK-EnvA cells were infected with RVΔG-mCherry at  
328 a multiplicity of infection (MOI) of 2. 24 hours later cells were washed 3 times in PBS and  
329 fresh media added, this was repeated 24 hours later. After 48 hours incubation, media was  
330 harvested, filtered and viral particles concentrated by centrifugation. The virus was  
331 resuspended in PBS and further concentrated with Amicon Ultra 100 kDa protein  
332 concentrators. Viral titres were assessed by serial dilution of the virus on 293-TVA cells and  
333 virus of titre  $1 \times 10^8$  I.U./ml used for injection.

334

### 335 **Spinal cord injection**

336 For rabies tracing experiments p9 *PV<sup>HTB</sup>*; *TRPV1<sup>HTB</sup>* and *TRPM8<sup>HTB</sup>* mice were  
337 anesthetized with isoflurane and placed on a stereotaxic frame. A skin incision in the back  
338 was made to expose the most caudal ribs to identify the lumbar spinal cord level 1. RVΔG-  
339 mCherry/EnvA was injected starting from 300 μm deep into the dorsal horn and going back

340 dorsally, in 6 steps consisting of 50 nl pulses every 50  $\mu\text{m}$  on the left side (400  $\mu\text{m}$  lateral  
341 from the midline) of the spinal cord using a 0.5  $\mu\text{l}$  Hamilton syringe mounted on a UMP3  
342 UltraMicroPump (WPI). Skin was then sutured with a nylon surgical suture. Animals were  
343 sacrificed 7 days after injection (p16).

344

### 345 **Perfusion**

346 Animals were anesthetized by intraperitoneal injection of 0.1 ml ketamine /xylazine  
347 mix per 10 g of weight (final concentrations: 120 mg/kg and 10 mg/kg, respectively) and  
348 checked for toe-pinch reflex before starting any procedure. Animals were first transcardially  
349 perfused with ice-cold PBS until the liver was cleared of blood, followed by ice-cold 4%  
350 PFA.

351

### 352 **Spinal cord dissection and tissue processing**

353 Spinal cords were exposed by ventral laminectomy. Tissue was post-fixed overnight  
354 in 4% PFA at 4°C. This was followed by three washes with ice-cold PBS for 5 minutes each  
355 and over night incubation in 30% sucrose in phosphate buffer (0.1M PB) at 4°C for  
356 cryoprotection. Samples were embedded in Optimal Cutting Temperature (O.C.T.,  
357 TissueTek) compound, frozen on dry ice and stored at -80°C.

358

### 359 **Immunohistochemistry**

360 Consecutive sections (30 $\mu\text{m}$  thick) were made with a Leica cryostat and mounted on  
361 Superfrost Plus slides (VWR). For immunohistochemical staining, sections were hydrated  
362 with 1X PBS for 20 minutes and permeabilized with 0.1% Triton X-100/PBS for 10 minutes  
363 at room temperature. Primary antibodies diluted in Triton X-100/PBS were incubated  
364 overnight at 4°C. Primary antibody dilutions were used as follows: rabbit anti-DsRed 1:1000

365 (Takara), goat anti-ChAT 1:250 (Surmeli et al., 2011), sheep anti-GFP 1:2000 (Bio-rad),  
366 chicken anti-PV 1:10000 (de Nooij et al., 2013), sheep anti-Chx10 1:500 (Abcam), rabbit  
367 anti-Calbindin 28k 1:2000 (SWANT), goat anti-FoxP2 1:250 (Santa Cruz), guinea pig anti-  
368 Lbx1 1:10000 (Muller et al., 2002), rabbit anti-Lhx1 1:10000 (Generated in the Jessell  
369 laboratory), rabbit anti-PKC $\gamma$  1:500 (Cell Signaling Technology), FITC conjugated-IB4  
370 (Sigma) and rabbit anti-CGRP 1:2000 (Immunostar). After washing 3 times with Triton X-  
371 100/PBS, sections were incubated with secondary antibodies for 1hour at room temperature.  
372 Alexa Fluor 488- and Cy3-conjugated secondary antibodies were used at 1:1000, Cy5-  
373 conjugated secondary antibodies at 1:500. Sections were then washed twice with 0.1% Triton  
374 X-100/PBS for 5 minutes each and once with 1X PBS for 10 minutes. Slides were  
375 coverslipped using Vectashield mounting medium. Images were acquired using confocal  
376 microscope (Zeiss LSM 800).

377

### 378 **Tissue clearing**

379 The dura, from a post-fixed spinal cord, was carefully and completely removed. The  
380 tissue was cleared as previously described (Susaki et al., 2015). Briefly, samples were  
381 incubated at 37°C in ½ Scale CUBIC 1 with water for 3-6 hours and then incubated with  
382 Scale CUBIC 1 overnight at 37°C. On the 2nd day, the Scale CUBIC 1 was changed with a  
383 fresh one and leaved for other 2 days. Then, samples were washed with 1X PBS overnight  
384 and incubated in ½ Scale CUBIC 2 in PBS for 3-6 hours at 37°C. The next day, samples were  
385 transferred in Pure Scale CUBIC 2 overnight at 37°C. After clearing, samples were  
386 immediately imaged in mineral oil with a Zeiss Z1 light sheet microscope.

387

### 388 **Neuronal position analysis**

389            Three-dimensional positional analysis was performed as previously described (Dewitz  
390 et al., 2018). Briefly, high-resolution images of the spinal cord were processed with the  
391 imaging software IMARIS using the “spots” function to assign Cartesian coordinates to all  
392 labeled neurons. We set the central canal as the 0, 0 coordinate for the medio-lateral (x-axis)  
393 and dorso-ventral (y-axis) axes. These coordinates (x and y) were rotated and normalized to a  
394 standardized spinal cord, whose dimensions were obtained by calculating the average size of  
395 spinal cords at p16 (M-L: 800  $\mu$ m, D-V: 600  $\mu$ m), to avoid variability in size and orientation  
396 of the spinal cord between experiments. Datasets were aligned on the z-axis by starting  
397 analysis from the section where the first labeled neurons appeared ( $z=0$ ) in the L1 segment  
398 and progressed caudally for more than 2 mm, covering two lumbar segments of the spinal  
399 cord. Positional analyses were performed using custom script in “R project” (R Foundation  
400 for Statistical Computing, Vienna, Austria, <http://www.r-project.org>). Contour and Density  
401 plot were generated using “ggplot2” package. The heat maps were used to compare the 2D  
402 spatial distribution of interneurons within each experiment and generated with the “corrplot”  
403 function. The similarity between experiments was measured by the Pearson correlation  
404 coefficient “r”.

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506 **Figure Legends**

507 **Figure 1. Retrograde infection of primary somatosensory neurons and anterograde**  
508 **monosynaptic spread into spinal neurons.**

509 A) Schematics representing the strategy for genetic targeting of G and TVA expression in  
510 DRG neurons and monosynaptic tracing with pseudotyped rabies injection in the spinal cord.

511 SN, sensory neurons; IN, interneurons; MN, motor neurons; 1, primary infection; 2,  
512 secondary infection.

513 B) Parvalbumin expression in GFP<sup>+</sup> sensory neurons in p9 *PV<sup>HTB</sup>* mice.

514 C) Specificity of genetic tracing with the *PV<sup>HTB</sup>* line expressed as a percentage of GFP<sup>+</sup>  
515 sensory neurons labeled by parvalbumin staining.

516 D) Rabies expression (mCherry) in GFP<sup>+</sup> sensory neurons after RVΔG-mCherry/EnvA  
517 injection in *PV<sup>HTB</sup>* mice.

518 E) Specificity of sensory neurons targeting expressed as a percentage of RV<sup>+</sup> sensory neurons  
519 labeled by nuclear GFP after RVΔG-mCherry/EnvA injection in *PV<sup>HTB</sup>* mice.

520 F) Efficiency of sensory neurons targeting expressed as a percentage of GFP<sup>+</sup> sensory  
521 neurons labeled by mCherry after RVΔG-mCherry/EnvA injection in *PV<sup>HTB</sup>* mice.

522 G) Rabies expression (mCherry) in spinal neurons at p16 after RVΔG-mCherry/EnvA  
523 injection in p9 *PV<sup>HTB</sup>* mice. Arrows points to motor neurons in the ventral spinal cord.

524 H) tdTomato labeling of proprioceptive sensory afferents in the spinal cord of *PV::Cre<sup>+/-</sup>;*  
525 *Ail4<sup>f/+</sup>* mice.

526

527 **Figure 2. Rabies tracing of spinal proprioceptive circuits.**

528 A) Rabies expression (mCherry) in spinal interneurons and ChAT<sup>+</sup> motor neurons after  
529 RVΔG-mCherry/EnvA injection in p16 *PV<sup>HTB</sup>* mice.

- 530 B) Digital reconstruction of RV<sup>+</sup> interneuron positions in *PV<sup>HTB</sup>* experiments. IN,  
531 interneurons.
- 532 C) Digital reconstruction of RV<sup>+</sup>; ChAT<sup>+</sup> motor neuron positions in *PV<sup>HTB</sup>* experiments. MN,  
533 motor neurons.
- 534 D) Dorso-ventral (top) and medio-lateral (bottom) density analyses of RV<sup>+</sup> interneurons (top)  
535 and RV<sup>+</sup>; ChAT<sup>+</sup> motor neurons (bottom) in three *PV<sup>HTB</sup>* experiments.
- 536 E) Number of starter cells defined as GFP<sup>+</sup>; RV<sup>+</sup> sensory neurons in *PV<sup>HTB</sup>* experiments.
- 537 F) Number of spinal neurons traced in *PV<sup>HTB</sup>* experiments. IN, interneurons; MN, motor  
538 neurons.
- 539 G) Connectivity index, the average number of second order neurons traced from a single  
540 starter cell in *PV<sup>HTB</sup>* experiments.
- 541 H) Correlation analysis of interneurons and motor neurons positional coordinates in *PV<sup>HTB</sup>*  
542 experiments (“IN vs IN” R ≥ 0.9; “MN vs MN” R ≥ 0.8). Scale bar indicates correlation  
543 values. IN, interneurons; MN, motor neurons.

544

545 **Figure 3. Rabies tracing of spinal thermosensitive circuits.**

- 546 A and B) Rabies expression (mCherry) in GFP<sup>+</sup> sensory neurons labeled after RVΔG-  
547 mCherry/EnvA injection in *TRPV1<sup>HTB</sup>* (A) and *TRPM8<sup>HTB</sup>* (B) mice.
- 548 C) Specificity of sensory neurons targeting expressed as a percentage of RV<sup>+</sup> sensory neurons  
549 labeled by nuclear GFP after RVΔG-mCherry/EnvA injection in *TRPV1<sup>HTB</sup>* and *TRPM8<sup>HTB</sup>*  
550 mice.
- 551 D) Efficiency of sensory neurons targeting expressed as a percentage of GFP<sup>+</sup> sensory  
552 neurons labeled by mCherry after RVΔG-mCherry/EnvA injection in *TRPV1<sup>HTB</sup>* and  
553 *TRPM8<sup>HTB</sup>* mice.

554 E and F) RV<sup>+</sup> (mCherry) spinal neurons after RVΔG-mCherry/EnvA injection in *TRPVI<sup>HTB</sup>*  
555 (E) and *TRPM8<sup>HTB</sup>* (F) mice.

556 G and H) Digital reconstruction of RV<sup>+</sup> interneuron positions in *TRPVI<sup>HTB</sup>* (G) and  
557 *TRPM8<sup>HTB</sup>* (H) experiments.

558 I) Number of starter cells defined as GFP<sup>+</sup>; RV<sup>+</sup> sensory neurons in *TRPVI<sup>HTB</sup>* and *TRPM8<sup>HTB</sup>*  
559 experiments.

560 J) Number of spinal neurons traced in *TRPVI<sup>HTB</sup>* and *TRPM8<sup>HTB</sup>* experiments.

561 K) Connectivity index defined as the average number of second order neurons traced from a  
562 single starter cell in *TRPVI<sup>HTB</sup>* and *TRPM8<sup>HTB</sup>* experiments.

563

564 **Figure 4. Organization of sensory circuits for mechanical and thermal sensation in the**  
565 **spinal cord.**

566 A) Transverse contour density plots and dorso-ventral distribution of post-sensory neurons in  
567 the dorsal (300 to 600μm), intermediate (0 to 300μm), ventral (0 to -600μm) and contralateral  
568 spinal cord of *PV<sup>HTB</sup>* (interneurons: black; motor neurons: green), *TRPVI<sup>HTB</sup>* (Red) and  
569 *TRPM8<sup>HTB</sup>* (Blue) experiments.

570 B) Correlation analysis of post-sensory neurons Cartesian coordinates in *PV<sup>HTB</sup>*, *TRPVI<sup>HTB</sup>*  
571 and *TRPM8<sup>HTB</sup>* experiments (“*TRPVI<sup>HTB</sup>* vs *TRPM8<sup>HTB</sup>*”,  $r \geq 0.85$ ; “*TRPVI<sup>HTB</sup>* or *TRPM8<sup>HTB</sup>*  
572 vs *PV<sup>HTB</sup>*”,  $r \leq 0.55$ ). Scale bar indicates correlation values.

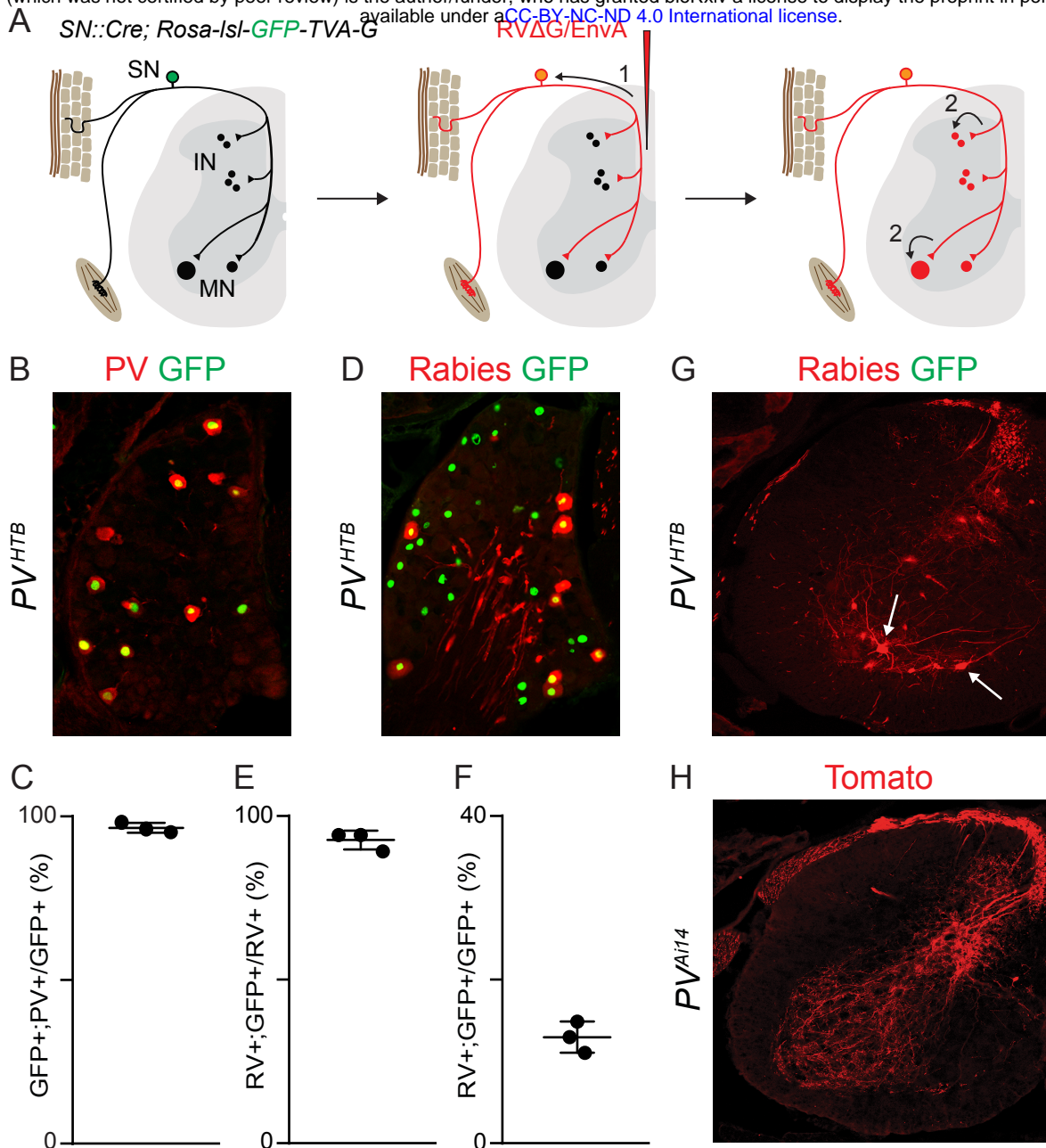
573 C) PKCγ and mCherry expression in ipsilateral dorsal spinal cords after RVΔG-  
574 mCherry/EnvA injection in *PV<sup>HTB</sup>*, *TRPVI<sup>HTB</sup>* and *TRPM8<sup>HTB</sup>* mice.

575 D) Digital reconstruction of RV<sup>+</sup> interneuron positions in the dorsal spinal cord of *PV<sup>HTB</sup>*  
576 (black), *TRPVI<sup>HTB</sup>* (red) and *TRPM8<sup>HTB</sup>* experiments.

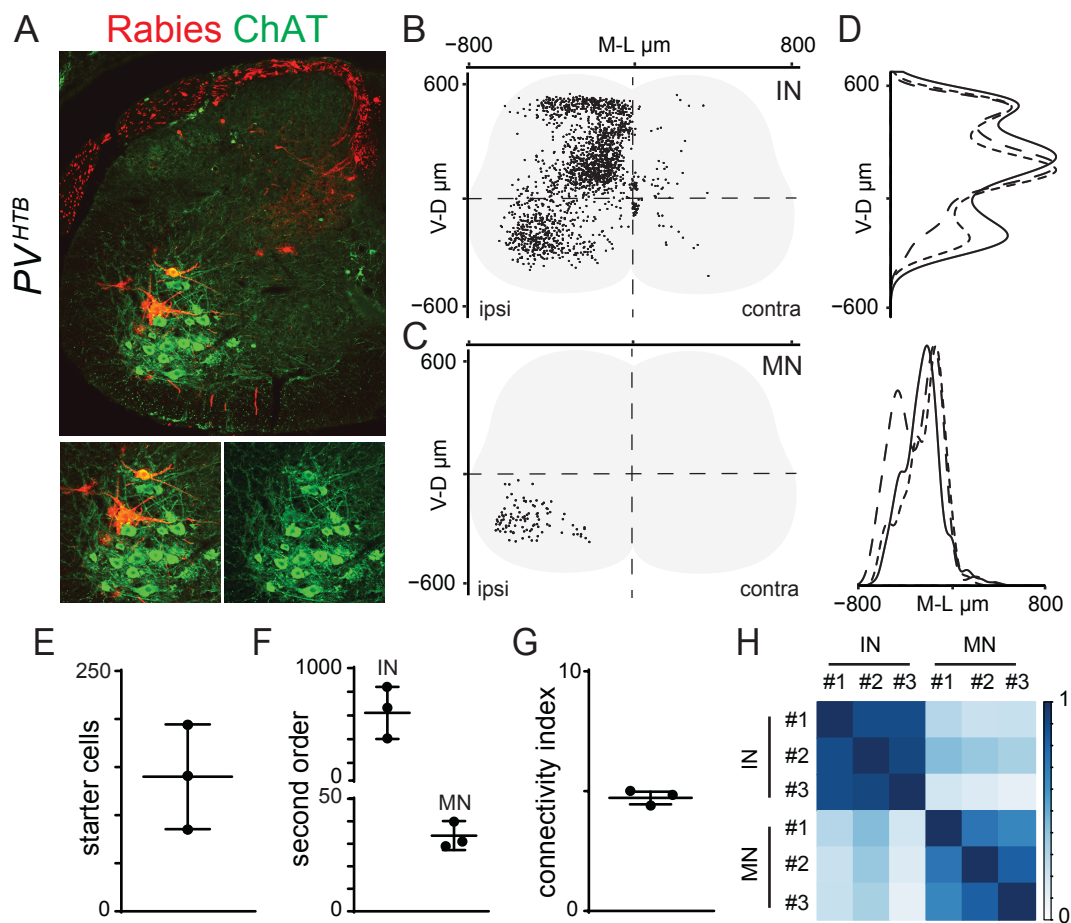
577 E) Box-plot showing dorso-ventral the distributions of RV<sup>+</sup> interneurons in the dorsal horn of  
578 *PV<sup>HTB</sup>* (black), *TRPVI<sup>HTB</sup>* (red) and *TRPM8<sup>HTB</sup>* (blue) experiments. PKC $\gamma$  staining (white) is  
579 used as an internal reference.

580 F) Dorso-ventral density plots showing the distributions of RV<sup>+</sup> interneurons in the dorsal  
581 horn of *PV<sup>HTB</sup>* (black), *TRPVI<sup>HTB</sup>* (red) and *TRPM8<sup>HTB</sup>* (blue) experiments.

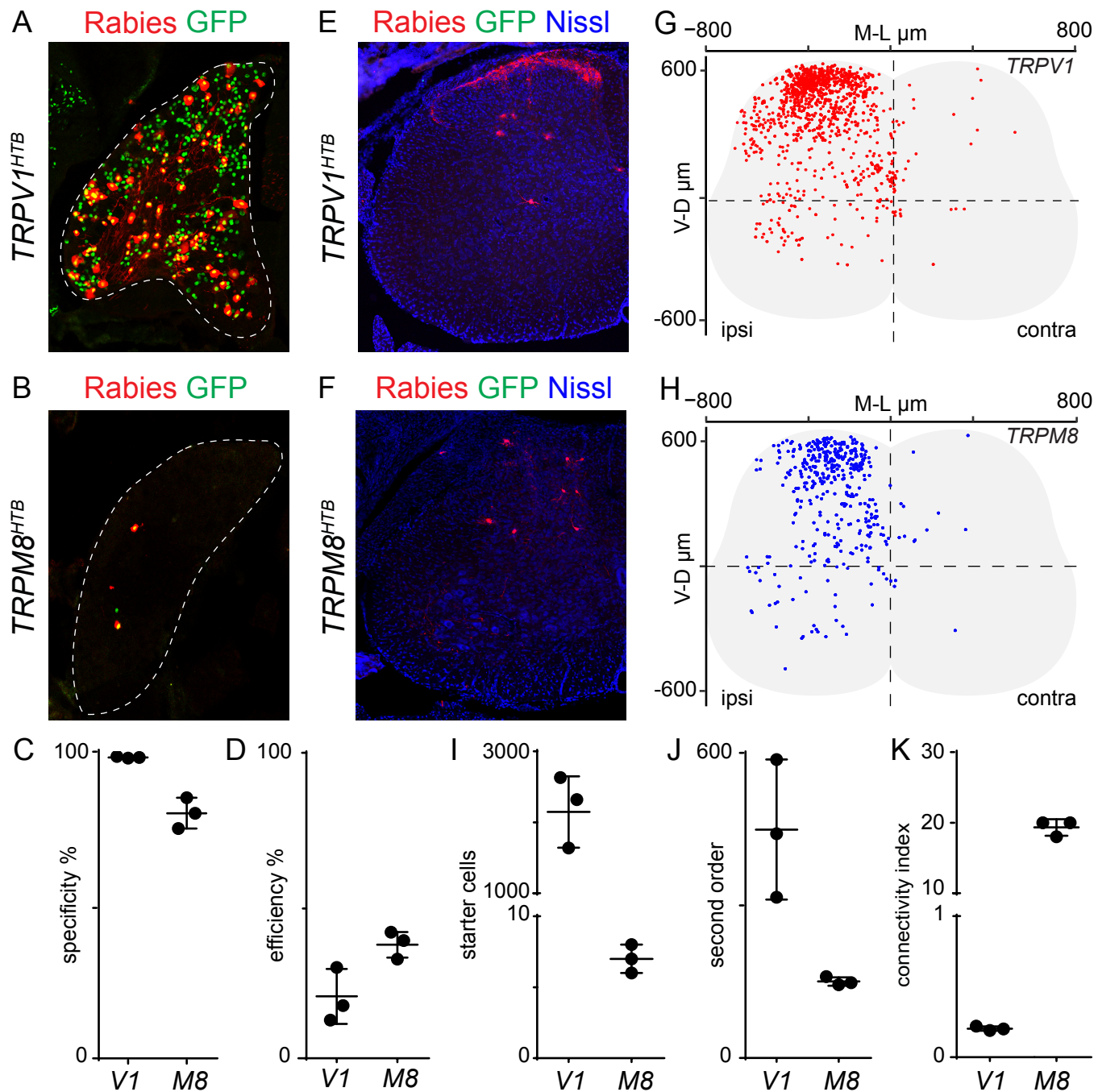
582 G) CGRP (laminae I and IIo), IB4 (lamina II<sub>m</sub>), and mCherry expression in ipsilateral dorsal  
583 spinal cords after RV $\Delta$ G-mCherry/EnvA L1 injection in p9 *TRPVI<sup>HTB</sup>* mice.



**Figure 1**

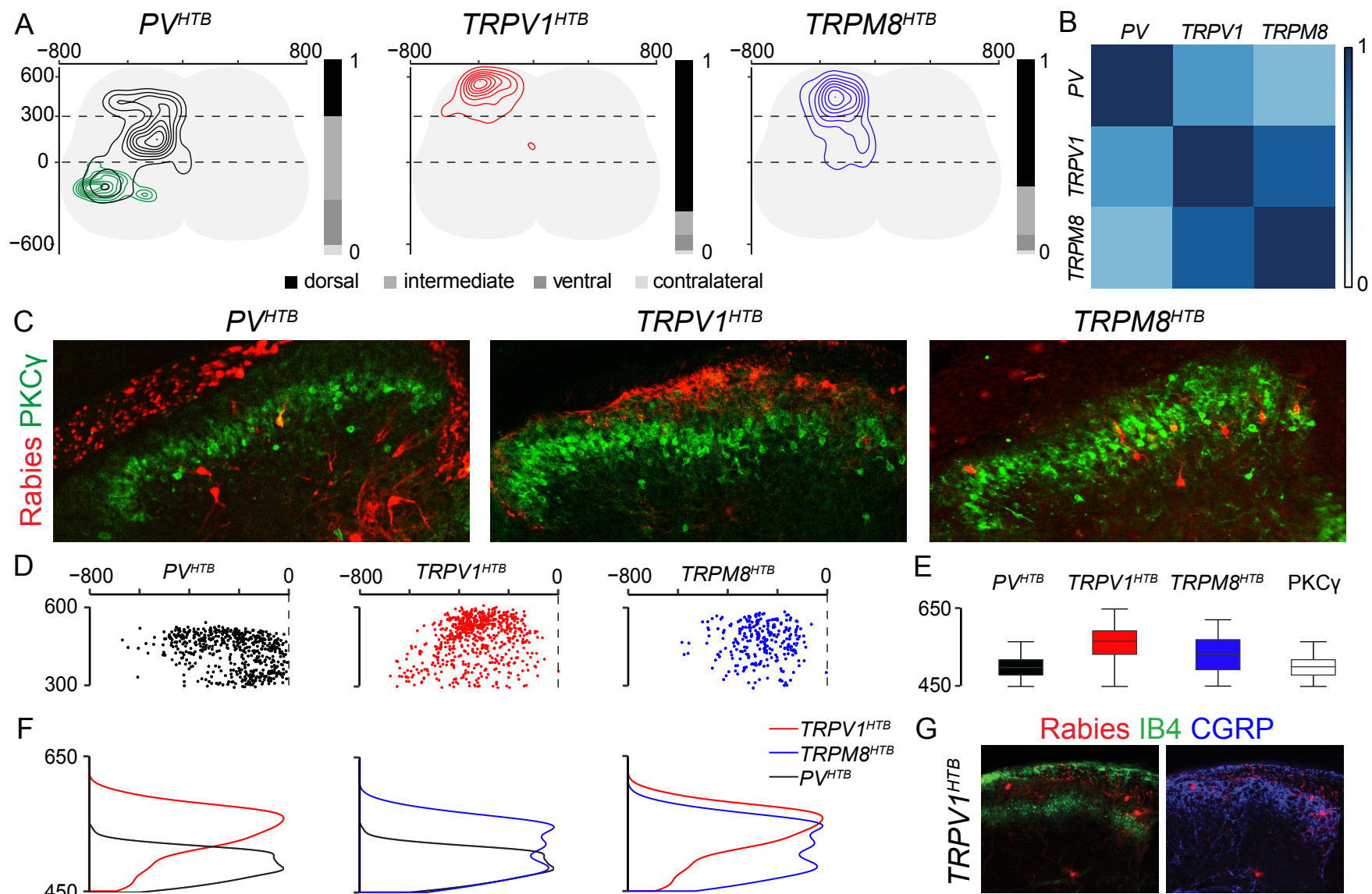


**Figure 2**



**Figure 3**





**Figure 4**