

1 **Distinct descending motor cortex pathways and their roles in movement**

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12

13 **ABSTRACT**

14

15 Activity in motor cortex predicts specific movements, seconds before they are initiated. This preparatory
16 activity has been observed in L5 descending ‘pyramidal tract’ (PT) neurons. A key question is how
17 preparatory activity can be maintained without causing movement, and how preparatory activity is
18 eventually converted to a motor command to trigger appropriate movements. We used single cell
19 transcriptional profiling and axonal reconstructions to identify two types of PT neuron. Both types share
20 projections to multiple targets in the basal ganglia and brainstem. One type projects to thalamic regions
21 that connect back to motor cortex. In a delayed-response task, these neurons produced early preparatory
22 activity that persisted until the movement. The second type projects to motor centers in the medulla and
23 produced late preparatory activity and motor commands. These results indicate that two motor cortex
24 output neurons are specialized for distinct roles in motor control.

25

26 INTRODUCTION

27 Motor cortex plays critical roles in planning and executing voluntary movements. Activity in motor
28 cortex anticipates specific future movements, often seconds before movement onset (reviewed in Refs
29 [1,2]). This dynamic neural process, referred to as preparatory activity, is thought to move the state of the
30 motor cortex to an initial condition appropriate for eliciting rapid, accurate movements³. In addition,
31 motor cortex activity is highly modulated during movement onset, consistent with commands that control
32 the timing and direction of movements^{4,5}.

33 Reconciling the dual roles of motor cortex requires an understanding of the cell types that make up the
34 cortical circuit, and how these cell types integrate into the multi-regional circuits that maintain short-term
35 memories and produce voluntary movements. Motor cortex comprises distinct cell types that differ in
36 their location, gene expression pattern, electrophysiology, and connectivity. Intratelencephalic (IT)
37 neurons in layers (L) 2-6 receive diverse input from other cortical areas and excite pyramidal tract (PT)
38 neurons⁶⁻⁸. PT neurons, whose somata define neocortical L5b⁹, are of particular significance as they make
39 the only long-range connections linking the motor cortex with premotor centers in the brainstem and
40 spinal cord¹⁰. PT neurons thus coordinate cortical and subcortical brain regions to produce behavior^{11,12}.
41 Lesioning PT axons causes persistent motor deficits^{12,13}. PT neurons also constitute a major component of
42 the cortical projection to the thalamus¹⁴⁻¹⁶. Previous studies have shown that preparatory activity is not
43 maintained by motor cortex in isolation, instead requiring reverberations in a thalamocortical loop¹⁷.
44 Consistent with roles in both movement planning and initiation, PT neurons show diverse activity
45 patterns, including preparatory activity and movement commands¹⁸⁻²¹. PT neurons are also structurally
46 heterogeneous, with complex projection patterns in the midbrain and hindbrain¹⁴.

47 Here we show that PT neurons in mouse motor cortex comprise two cell types with distinct gene
48 expression profiles and projection patterns. We refer to these cell types as PT^{upper} and PT^{lower} neurons,
49 reflecting their distributions in different sublaminae in L5b. PT^{upper} project to the thalamus, which forms a
50 feedback loop with motor cortex. PT^{lower} neurons project to premotor centers in the medulla. Cell type-
51 specific extracellular recordings in the anterior lateral motor cortex (ALM) during a delayed-response
52 task suggest that PT^{upper} neurons are involved in motor planning, whereas PT^{lower} neurons play roles in
53 movement execution. Thus, motor cortex coordinates its two complementary roles at the level of distinct
54 cell types.

55

56 RESULTS

57 Two types of PT neurons in Layer 5

58 Single-cell RNA sequencing (scRNA-Seq) was used to produce a taxonomic classification of cell types²²
59 in the anterior lateral motor cortex (ALM) and in primary visual cortex (V1). A total of 21,749 scRNA-
60 Seq transcriptomes were collected, including 9,035 from ALM. Dimensionality reduction was used to
61 extract features from single-cell transcriptomes²³, which in turn were the basis for clustering²². This
62 procedure identified 116 transcriptomic clusters. GABAergic neurons partitioned into 49 clusters, all of
63 which contained neurons from both ALM and V1²². ALM glutamatergic neurons belonged to 21 clusters
64 (**Fig. 1**), which were distinct from the glutamatergic clusters identified in V1.

65 ALM and visual cortex perform different computations. Visual cortex and other sensory cortical areas
66 process sensory information with millisecond time-scale dynamics^{24,25}. ALM shows slow dynamics
67 related to short-term memory and motor planning, in addition to fast dynamics related to the initiation of
68 orofacial movements^{18,26}. To gain an understanding of the neural circuit specializations underlying ALM
69 function, we analyzed ALM projection neurons. Transcriptomically, ALM glutamatergic projection
70 neurons within the same cortical layer and/or belonging to the same projection types were generally more
71 similar to each other. Expression clusters corresponding to L2/3 intratelencephalic (IT), L5/6 IT, L5
72 pyramidal tract (PT), L6 corticothalamic (CT), and L6b subplate neurons exhibited a higher degree of
73 similarity within a projection type than between types (**Fig. 1a,c**), as did L5 neurons that lack long-range
74 projections (NP; ‘near projecting’).

75 PT neurons form the sole cortical projection to motor areas in the midbrain and hindbrain, and therefore
76 likely play important roles in motor planning and execution. For the rest of this study, we focus on these
77 neurons. ALM PT neurons, retrogradely labeled from a diverse set of PT targets²², mapped to three
78 distinct transcriptomic clusters: the *Rgs8* and the closely related *Hpgd* and *Htr2c* clusters (**Fig. 1** and
79 **EDFig. 1a**). To map the structural diversity of PT neurons, we imaged and reconstructed the brain-wide
80 axonal projections of entire PT neurons, labeled randomly by viral injection in ALM²⁷ (n = 12; **Fig. 2a,b**
81 and **EDTable 1**; median axonal length: 121,037 μm , range: 80,873 - 188,105 μm ; median branch points:
82 243, range: 144 - 540). Single-neuron reconstructions suggested two classes of PT neurons based on their
83 axonal projections. Axons of one group innervated the thalamus (n=8; **Fig. 2a,b**; *yellow-green hues* and
84 **EDFig. 2**). The other group bypassed the thalamus and branched extensively in the reticular nuclei of the
85 medulla (n=4; **Fig. 2a,b**; *red-brown hues* and **EDFig. 2**).

86 To determine the spatial distribution of these projection types in motor cortex, neurons were retrogradely
87 labeled from the thalamus and medulla using AAVretro²⁸. Thalamus-projecting PT neurons were located
88 in upper L5b (**Fig. 2c,d** and **EDFig. 4**; *green cells*) and medulla-projecting PT neurons in lower L5b (*red*
89 *cells*). This pattern was similar across all of motor cortex, including both primary and secondary motor
90 areas. Retrograde labeling from the superior colliculus (SC) and pons labeled PT neurons across both L5b
91 sublaminae, with pons-projecting neurons concentrated somewhat in the deeper sublayer (**Fig. 2c**).
92 Consistent with their laminar distributions, few neurons (2.2%; 22/984) were co-labeled by injections into
93 the thalamus and medulla. This lack of overlap did not result from inefficient retrograde labeling; in
94 experiments in which neurons were retrogradely labeled from both the thalamus and SC, most PT neurons
95 (77.1%; 687/890) projecting to the thalamus were co-labeled.

96 To link projection classes and transcriptomic clusters, we examined gene expression in medulla-
97 projecting and thalamus-projecting PT neurons, isolated from AAVretro-labeled brains (Methods). All
98 (63/63) medulla-projecting PT neurons mapped to the *Rgs8* taxonomic cluster. Similarly, thalamus-
99 projecting PT neurons mapped to the *Htr2c* and *Hpgd* clusters (88/94; *Htr2c*, 33/94; *Hpgd*, 55/94) (**Fig.**

100 **1c, 2e,f**). Furthermore, all thalamus-projecting and medulla-projecting PT neurons (157/157) could be
101 separated using a single linear boundary in t-SNE space (**Fig. 2e**; *dotted line*). PT neurons retrogradely
102 labeled from the pons and SC mapped to the same PT clusters (SC: $n=92$ total; 44 *Rgs8*, 20 *Htr2c*, 28
103 *Hpgd*; pons: $n=100$ total; 88 *Rgs8*, 9 *Htr2c*, 3 *Hpgd*; **Fig. 2f**).

104 Axonal reconstructions and transcriptomic data suggest that PT neurons can be divided into two distinct
105 cell types in motor cortex. To determine if thalamus-projecting and medulla-projecting PT neurons
106 account for the majority of PT neurons in motor cortex, we examined additional PT neurons that were
107 reconstructed partially (thalamus-projecting: $n=3$; medulla-projecting: $n=3$). All (18/18) partially and
108 fully reconstructed PT neurons projected to the SC and no (0/18) PT neurons lacked projections to both
109 the thalamus and medulla (**EDTable 1**). These results suggest that most – if not all – PT neurons are
110 accounted for by the medulla-projecting and thalamus-projecting types. We refer to the superficial,
111 thalamus-projecting *Htr2c/Hpgd* cell type as PT^{upper} neurons and the deep, medulla-projecting *Rgs8* cell
112 type as PT^{lower} neurons, reflecting their laminar distributions.

113 Cell type-specific markers

114 We combined scRNA-Seq with bulk RNA-Seq to identify marker genes for PT^{upper} and PT^{lower} cells. Bulk
115 RNA-Seq data was collected for PT^{upper} and PT^{lower} neurons from AAVretro-labeled brains (50-70
116 cells/sample; 6 replicates each; **Methods**). Expression levels in scRNA-Seq and bulk RNA-Seq were
117 highly correlated (Pearson's $R = 0.86-0.88$; **EDFig. 5**). Approximately 11,000 genes were detected per
118 cell type in bulk RNA-Seq, compared to approximately 10,000 in the scRNA-Seq data (median of
119 detected genes for single cells: PT^{upper} 9936 genes; PT^{lower} 9865 genes). Differentially expressed (DE)
120 genes identified from scRNA-Seq were also differentially expressed in bulk RNA-Seq (**EDFig. 6a**).
121 Conversely, DE genes from bulk RNA-Seq (**EDFig. 6b**) displayed consistent relative expression levels in
122 scRNA-Seq (**EDFig. 1b**). Differentially expressed transcripts (**Fig. 3a**) were examined in the Allen Brain
123 Atlas (<http://mouse.brain-map.org>) for enrichment in L5. Two transcripts, *Npnt* and *Slco2a1*, were
124 confirmed as cell-type specific markers with single-molecule RNA fluorescence *in situ* hybridization
125 (smFISH; **Fig. 3b,c**). PT^{lower} neurons expressed higher levels of *Slco2a1* mRNA (inter-quartile range, IQR
126 = 9-28 puncta) than PT^{upper} neurons (IQR = 0-3 puncta). In contrast, PT^{upper} neurons expressed higher
127 levels of *Npnt* (IQR = 15-30 puncta) than PT^{lower} neurons (0-4 puncta).

128 Cell type-specific recordings

129 The projection patterns of the PT cell types suggest distinct roles in motor control. The cortico-
130 thalamocortical loop is necessary for maintaining persistent preparatory activity related to motor
131 planning¹⁷. PT^{upper} cells project to the thalamus and lack projections to premotor nuclei in the medulla.
132 These characteristics suggest a role for PT^{upper} cells in generating and/or maintaining preparatory activity.
133 In contrast, PT^{lower} cells project to premotor centers in the medulla and the spinal cord (**Fig. 2a,b** and
134 **EDFigs. 2,3**), with few collaterals in the basal ganglia and thalamus, suggesting a role in movement
135 execution.

136 We performed projection-specific recordings in ALM during a delayed-response task^{20,26} (**Fig. 4a,b**).
137 Mice were trained to discriminate object locations with their whiskers²⁶ and signal their decision about
138 object location with skilled, directional licking ('left / right'), but only after a delay epoch lasting 1.3
139 seconds. The delay epoch was terminated by an auditory 'go' cue instructing animals to respond. ALM is
140 a hub for planning and executing movements in this task^{18,26,29,30}.

141 PT^{upper} or PT^{lower} cells were infected with AAVretro expressing channelrhodopsin-2 (ChR2). Fiber optic
142 cannulae were implanted into the thalamus (to activate PT^{upper} cells; **Fig. 4c, top**) or medulla (to activate

143 PT^{lower} cells; **Fig. 4c, bottom**). Chr2-expressing cells in ALM were identified with axonal
144 photostimulation and extracellular recordings in ALM using a collision test (61 PT^{upper} cells, 8 mice; 69
145 PT^{lower} neurons, 4 mice; **Fig. 4d and EDFig. 7**)¹⁸. Identified PT^{upper} cells were at more superficial
146 recording depths than PT^{lower} neurons, consistent with the retrograde labeling experiments (**Fig. 4e** and
147 **EDFig. 4**). Layer 6 corticothalamic cells, which also innervate the thalamus, were inefficiently labeled by
148 AAVretro (**Fig. 2c,d** and **EDFig. 4**)²⁸ and excluded based on recording depth, several hundred
149 micrometers deeper than the PT^{upper} cells. Baseline and trial-averaged peak spike rates were not
150 significantly different ($p > 0.1$, two-sample t-test) in PT^{upper} cells (baseline: median= 4.3 ± 3.5 Hz; peak:
151 median= 17.7 ± 13.8 Hz) and PT^{lower} cells (baseline: median= 5.2 ± 4.8 Hz; peak: median= 17.2 ± 22 Hz).
152 Spike rates in PT^{lower} cells were more heterogeneous across the population (baseline: $p = 0.02$; peak: $p =$
153 3×10^{-4} ; χ^2 test). A substantial proportion of PT^{lower} neurons displayed spike bursts (18.8%), which were
154 rare among PT^{upper} cells (3.3%; $p = 0.006$, Fisher's exact test; **EDFigs. 7,8**).

155 Preparatory activity

156 Individual neurons exhibited diverse patterns of activity and selectivity, defined as the difference in spike
157 rate between trial types ("lick left" vs "lick right") (**Fig. 4f**). Most recorded PT neurons had significant
158 selectivity ($p < 0.01$, two-sided Mann-Whitney U-test) during at least one task epoch (122/130; 94%). A
159 subset displayed selectivity that emerged at the start of the sample epoch and persisted through the delay
160 epoch until the response epoch (**Figs. 4f; left cells**), suggesting that a subset of PT neurons stably encode
161 upcoming movement direction. This preparatory activity is a form of short-term memory that links past
162 events and future movements.

163 We investigated the emergence and maintenance of preparatory activity in populations of PT^{upper} and
164 PT^{lower} cells. We analyzed population dynamics in activity space, where each dimension corresponds to
165 the activity of one neuron. Preparatory activity for different movement directions corresponded to distinct
166 trajectories in the high-dimensional activity space. For each population, we computed the linear
167 combination of cells that best discriminated trial type during the first 400 ms of the sample epoch (CD_{early}:
168 'early coding dimension'; **Fig. 5a,b**). We then projected the trial-averaged activity of all cells in the
169 population along CD_{early} (**Fig. 5a**) to produce the one-dimensional projection of each activity space
170 trajectory with maximal selectivity during this time period. In this projection, selectivity was larger and
171 more consistent across trials in the PT^{upper} population compared to the PT^{lower} population (**Fig. 5b**).
172 Furthermore, selectivity in the PT^{upper} population remained constant throughout the sample and delay
173 epochs and until the go cue. We conclude that the PT^{upper} population retained decision-related information
174 for the duration of the behavioral trial. In contrast, selectivity in the PT^{lower} population decayed rapidly
175 along CD_{early} and was lost at the time of the go cue (no significant selectivity; $p = 0.15$, bootstrap). This
176 decay did not reflect a lack of any selectivity in the PT^{lower} population; along a different dimension in
177 activity space that maximized selectivity at the end of the delay epoch, CD_{late}, selectivity was substantial
178 in both cell types (**EDFig. 9**; PT^{upper}: 40/61; PT^{lower}: 44/69)¹⁸. We computed the stability of the coding
179 dimension across trial time. For the PT^{upper} population, the CD remained similar across the sample and
180 delay epochs, whereas for the PT^{lower} population, the CD was uncorrelated across epochs (**EDFig. 10**). As
181 suggested by population analyses, individual PT^{upper} neurons displayed persistent selectivity throughout
182 the sample and delay epochs (**Fig. 5c**) and decoded trial type significantly better than PT^{lower} neurons
183 during the sample epoch (**EDFig. 11**).

184 Movement commands

185 The rhythmic movements involved in licking and swallowing are coordinated by circuits in the reticular
186 nuclei of the medulla^{31,32}. Microstimulation of ALM is sufficient to initiate directional licking^{18,33}. PT^{lower}

187 neurons provide a direct path from ALM to the premotor circuits in the medulla (**Fig. 2a,b** and **EDFigs.**
188 **2,3**). We reasoned that putative ALM signals driving movement should have selectivity for movement
189 and emerge before movement onset. In addition, movement commands should lie along a dimension in
190 activity space that is orthogonal to the dimension that predicts movement in the delay epoch; otherwise
191 movement would be triggered before the go cue^{34,35}.

192 For each population, we determined CD_{go} as the dimension that maximizes selectivity immediately after
193 the go cue (400 ms), orthogonal to CD_{late} (**Fig. 6a-c**). Along CD_{go} , selectivity was larger, emerged faster,
194 and persisted longer in the PT^{lower} population compared to PT^{upper} cells (**Fig. 6 b,c**). In the PT^{lower}
195 population, significant selectivity emerged 24 ms following the go cue, faster than in the PT^{upper}
196 population (46 ms) (**Fig. 6c**). The onset of the first detectable movement occurred approximately 50 ms
197 after go cue onset (99% confidence interval = 38-56 ms). The coding dimension changed rapidly at the go
198 cue in the PT^{lower} population, and more slowly, over several hundred milliseconds, in the PT^{upper}
199 population (**EDFig. 10**). Individual PT^{lower} neurons displayed pronounced changes in selectivity at the go
200 cue (**Fig. 6d**).

201 Each bout of licking consists of a sequence of directional tongue protrusions at a stereotyped frequency
202 (approximately 8 Hz) (**Fig. 4,b**). Aligning PT^{lower} activity to the last lick in a bout revealed additional
203 movement-related features. Selectivity along both the CD_{late} and the CD_{go} dimensions ceased with the
204 offset of movement (**EDFig. 12a,b**), simultaneous with an abrupt change in the coding dimension
205 (**EDFig. 12c**). This transition was not observed in the dynamics of the PT^{upper} population (**EDFig. 12a-c**).
206 Indeed, examining the activity of single neurons in the PT^{lower} population revealed neurons that were
207 strongly modulated at the go cue, at the offset of movement, or both (**EDFig. 12d**). These results show
208 that subgroups of PT^{lower} neurons have activity patterns consistent with roles in initiating and/or
209 terminating movements.

210

211 DISCUSSION

212 Pyramidal tract (PT) neurons of motor cortex exhibit diverse activity patterns that are related to
213 movement planning and execution^{3,5,18,20,35}. We have shown that PT neurons in motor cortex comprise at
214 least two cell types with distinct gene expression patterns, axonal projections, and specialized roles in
215 motor control. PT^{upper} neurons connect with the thalamus and avoid motor centers in the medulla. PT^{upper}
216 neuron activity represents a short-term memory that links sensory information and motor planning. PT^{lower}
217 neurons avoid the thalamus and project to motor nuclei in the medulla. These neurons appear to control
218 movement initiation and termination. PT neurons segregate into PT^{upper} and PT^{lower} types across the
219 entirety of motor cortex (**EDFig. 4**). This organizing principle may extend to non-motor cortical areas and
220 other mammals³⁶⁻³⁸.

221 Previous anatomical studies have suggested that collaterals of PT neurons projecting to motor centers also
222 innervate the thalamus^{14,39}, possibly providing an efference copy of motor commands for predicting the
223 sensory and motor consequences of self-movement¹⁵. We show that neurons that project to motor centers
224 do not project to the thalamus. Instead corticothalamic PT^{upper} neurons play more cognitive roles in motor
225 preparation. The thalamus could still process efference copies of motor commands through an indirect,
226 tectal pathway^{40,41}. The thalamus also receives a projection from L6 corticothalamic neurons, but these
227 neurons are sparsely active and uncoupled from PT neurons¹⁶. In addition, their weak synapses are
228 thought to play a modulatory role in thalamic excitation¹⁵.

229 Cell type-specific recordings link representations of information with the neural circuit diagram, which is
230 critical to understand the mechanisms of neural computation. The two PT neuron types express distinct
231 behavior-related signals, implying that only a subset of the information represented in a cortical region is
232 communicated to specific downstream structures^{18,42,43}. Preparatory activity appeared early, and was
233 persistent, in PT^{upper} neurons, whereas movement commands were observed in PT^{lower} neurons. At the
234 same time, multiple signals were multiplexed within the population of PT^{lower} neurons. Preparatory
235 activity emerged in this population during the delay epoch (along CD_{late}) and persisted through the go cue
236 and up to the termination of licking bouts. In the same cell type, and sometimes in the same individual
237 cells (e.g. **Fig 6d**; *Cell #3*), activity was strongly modulated following the go cue along a different
238 dimension (CD_{go}), consistent with a movement command. A complete description of neural coding
239 therefore requires measurements of neural populations of defined cell types.

240 In this study we restricted our analysis to ALM PT neurons and found two principal cell types. PT^{upper}
241 neurons corresponded to two transcriptomic clusters (**Fig. 1**), separated by many differentially expressed
242 genes (**Fig. 3a** and **EDFigs. 1,6**). Future studies linking detailed anatomy with transcriptional profiling
243 might lead to further subdivision of these types. In addition to PT neurons, ALM harbors ten
244 transcriptomic clusters corresponding to diverse IT neurons, which project to other cortical areas and the
245 striatum²² (**Fig. 1**). A subset of these neurons connect the two ALM hemispheres via the corpus callosum,
246 which is critical for robustness in preparatory activity³⁰. Identifying their roles in movement will require
247 experiments similar to those presented here, in addition to mapping the connections between these cell
248 types.

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361

362 FIGURE LEGENDS

363 **Figure 1. Taxonomy of motor cortex glutamatergic neurons based on single-cell RNA-seq. a.**
364 Hierarchical clustering of gene expression. Three gene expression clusters, identified by the genes *Rgs8*,
365 *Htr2c*, and *Hpgd*, correspond to pyramidal tract neurons. **b.** Constellation diagram of gene expression
366 clusters in ALM. Node diameters indicate the number of neurons belonging to each cluster (core) and
367 edges represent cells shared by two clusters (intermediates). **c.** Two-dimensional stochastic neighbor
368 embedding (tSNE) projection of transcriptomic data of sequenced single neurons in ALM. The cluster
369 memberships of individual neurons are color-coded as in panels (a) and (b).

370 **Figure 2. Two types of PT neuron in motor cortex. a.** Example dendritic and axonal reconstructions of
371 four single PT neurons. Two PT neurons project to the thalamus (*top; yellow-green hues*) and two project
372 to the medulla (*bottom; red-brown hues*). **b.** Reconstructions of 4 thalamus-projecting PT neurons and 4
373 medulla-projecting PT neurons overlaid and collapsed in the sagittal (top) and horizontal (bottom) planes.
374 Dendrites are denoted by thicker line segments. **c.** Nuclei of neurons retrogradely labeled from different
375 PT targets are located in distinct sublaminae of L5b. **d.** Nuclei of PT neurons retrogradely labeled from
376 the thalamus (*green*) or the medulla (*red*). **e.** Gene expression of PT neurons retrogradely labeled from the
377 thalamus (*green*) and medulla (*red*) in tSNE space (as in Fig. 1c). PT neurons projecting to the medulla
378 belong to the *Rgs8* cluster, whereas thalamus-projecting PT neurons were part of the *Htr2c* and *Hpgd*
379 clusters. **f.** Proportion of neurons retrogradely labeled from each PT target that were clustered into the
380 *Rgs8* and *Htr2c/Hpgd* expression clusters.

381 **Figure 3. Cell type markers. a.** Differentially expressed genes from scRNA-seq, represented by violin
382 plots. Each row represents a single gene, and values within rows are normalized between 0 and the
383 maximum expression value for each gene (right edge of each row; FPKM) and displayed on a \log_{10} scale.
384 Median values are shown as black dots within each violin. Differentially expressed genes are grouped by
385 the transcriptomic clusters that they differentiate. ‘Others’ refers to ALM non-PT expression clusters. **b.**
386 Single-molecule fluorescence *in situ* hybridization validating cell type-specific marker expression. Four
387 example fields of view are shown for each gene and projection class. Neurons labeled from the thalamus
388 or medulla are shown in red and RNA puncta are in green. **c.** Number of RNA puncta counted in cells of
389 each type. Number of cells: PT^{upper}: 50 *Npnt*, 66 *Slco2a1*; PT^{lower}: 39 *Npnt*, 56 *Slco2a1*.

390 **Figure 4. Cell type-specific extracellular neurophysiology. a.** Mice were trained on a delayed-response
391 task. On each trial, an object appeared within reach of the whiskers in one of two rostro-caudal positions
392 during the sample epoch (1.0 s). The pole was removed and after a brief delay epoch (1.3 s), mice
393 reported the pole position by licking a reward port on the right (caudal pole position) or the left (rostral
394 pole position). **b.** Performance during an example session. Dots represent licks to the right (*blue*) or the
395 left (*red*). Gray and black marks indicate correct and incorrect trials, respectively. **c.** Schematic for
396 stimulation and recording configuration for each cell type. **d.** Collision test for an example neuron. Trials
397 with spontaneous spikes preceding the light-evoked spike are shown binned by the latency of spikes
398 preceding the stimulus from top to bottom. Putative photostimulation-evoked spikes are at $+\Delta t$. When a
399 spike occurs in the interval $[-\Delta t, +\Delta t]$, a collision occurs with the photostimulation-evoked spike in the
400 axon and the spike at $+\Delta t$ is absent. **e.** Depth distribution of PT^{upper} and PT^{lower} neurons based on
401 micromanipulator readings. Depths are measured from the dorsal surface and are uncorrected for the
402 curvature of cortical layers. Error bars represent mean \pm s.d. **f.** Example identified neurons. *Top*: spike
403 rastergrams for correct lick right trials (*blue*) and lick left trials (*red*). *Bottom*: trial-averaged spike rates.
404 S, sample; D, delay; R, response.

405 **Figure 5. Persistent preparatory activity in PT^{upper} neurons.** **a.** Time-course of the linear combination
406 of neuronal activity that best differentiates trial type after stimulus onset (CD_{early}) on lick right (*blue*) and
407 lick left (*red*) trials for PT^{upper} (*top*) and PT^{lower} (*bottom*) neurons. **b.** Difference in CD_{early} projections on
408 lick right and lick left trials (selectivity) in PT^{upper} (*top; green*) and PT^{lower} (*bottom; orange*) neurons. **c.**
409 Example identified PT^{upper} neurons. *Top:* raster plots for correct lick right trials (*blue*) and lick left trials
410 (*red*). *Bottom:* trial-averaged spike rates. Shaded regions in (a) and (b) represent 95% confidence intervals
411 around the mean using hierarchical bootstrapping.

412 **Figure 6. Movement commands in in PT^{lower} neurons.** **a.** Time-course of the linear combination of
413 neuronal activity that best differentiates trial types after the go cue (CD_{go}) on lick right (*blue*) and lick left
414 (*red*) trials for PT^{upper} (*top*) and PT^{lower} (*bottom*) neurons. **b.** Difference in CD_{go} projections on lick right
415 and lick left trials (selectivity) in PT^{upper} (*top; green*) and PT^{lower} (*bottom; orange*) neurons. **c.** Data from
416 (b) expanded around the go cue. Gray region indicates the distribution of session-averaged reaction times
417 (earliest detected orofacial movement, *R.T.*; 99% confidence interval = 38-56 ms). Along the CD_{go} ,
418 selectivity in PT^{lower} neurons emerged 24 ms following the go cue, faster than in the PT^{upper} population (46
419 ms) and consistent with a role in movement initiation. **d.** Example identified PT^{lower} neurons. *Top:* raster
420 plots for correct lick right trials (*blue*) and lick left trials (*red*). *Bottom:* trial-averaged spike rates. Shaded
421 regions in (a-c) represent 95% confidence intervals around the mean using hierarchical bootstrapping.

422

423 EXTENDED DATA FIGURE LEGENDS

424 **Extended data figure 1. Differentially expressed genes in single cell RNA-seq PT neuron expression.**

425 **a.** Heat map of expression of differentially expressed genes. Columns in the heat map represent individual
426 cells, grouped by cluster (*Rgs8*, $n=209$; *Htr2c*, $n=69$; *Hpgd*, $n=90$). Rows represent genes selected by the
427 clustering algorithm to represent transcriptomic branch points between clusters. Color shows transcript
428 expression in fragments per kilobase of transcript per million mapped reads (FPKM), shown on a log-
429 scale. Pure blue shows cells with 0 transcripts of a given gene; pure red shows cells with maximal
430 expression (approximately 300 FPKM). **b.** Expression data for the same cells in (a) for genes identified
431 from bulk RNA-Seq as differentially expressed between the *Rgs8* and *Hpgd/Htr2c* clusters.

432 **Extended data figure 2. Distribution of axonal projections. a.** Axonal length within thalamic and
433 medullary targets for thalamus-targeting PT neurons ($n = 8$; *green*) and medulla-targeting PT neurons ($n =$
434 4 ; *orange*). **b.** Axonal lengths within other selected PT targets. **c.** Axonal termini within thalamic and
435 medullary targets. **d.** Axonal termini within other selected PT targets.

436 **Extended data figure 3. Anterograde anatomy: targets of PT^{upper} and PT^{lower} populations.** Groups of
437 cortical neurons were labeled from the thalamus (PT^{upper}; Mouse #1), the medulla (PT^{lower}; Mouse #2), or
438 both targets (Mouse #3) using AAVretro expressing spectrally-distinct fluorescent proteins. Top,
439 schematics of the labeling procedures. Left, rostro-caudal level (relative to Bregma). Right, imaged area is
440 indicated on annotated coronal sections taken from the Allen Reference Atlas at the corresponding rostro-
441 caudal level. Both types extended axon collaterals to motor-related SC, but to different parts. Axons from
442 PT^{upper} cells were apparent throughout all SC layers, with a particularly dense projection to the
443 ventrolateral aspect, whereas PT^{lower} neurons were restricted to the ventral superior colliculus and were
444 concentrated more caudally. Both groups projected to the pons, particularly the pontine gray, but with
445 terminations in largely non-overlapping zones. PT^{upper} cells exclusively projected to the globus pallidus
446 external segment and broadly targeted the dorsal, lateral and ventral striatum. PT^{lower} cells projected
447 sparsely to the lateral striatum. PT^{lower} neurons projected to the central amygdala and parasubthalamic
448 nucleus, although these projections arise from cortical neurons outside of ALM (not shown). PT^{lower}
449 neurons also made up the majority of the projection to the red nucleus, parabrachial nucleus, substantia
450 nigra pars compacta, motor and sensory trigeminal nuclei in the hindbrain, and the pyramidal tract. Both
451 cell types extended axon collaterals locally within the same sublamina as their somata, the subthalamic
452 nucleus, zona incerta, and the midbrain reticular nucleus. PT^{upper} cells appeared to project more broadly to
453 layer 1 in motor cortex. Mouse #1 and #2 were used for electrophysiological recordings and in both cases
454 projections are labeled with Chr2.

455 **Extended data figure 4. Distribution of thalamus- and medulla-projecting PT neurons. a.** The nuclei
456 of PT neurons were labeled from the thalamus (green cells) and medulla (red cells) using AAVretro.
457 Thalamus-projecting PT neurons are in upper L5b throughout motor cortex, whereas medulla-projecting
458 PT neurons are in deep L5b. Schematics to the left of each image set are annotated coronal sections
459 (Allen Reference Atlas) at approximately the same rostro-caudal level. **b.** AAVretro injection sites in the
460 thalamus (*left*) and medulla (*right*).

461 **Extended data figure 5. Comparison of single cell RNA-Seq and bulk RNA-Seq. a.** Scatter plot of
462 PT^{upper} gene expression (measured as FPKM + 1, shown in log-scale) from scRNA-Seq (x-axis) and bulk
463 RNA-Seq (y-axis) ($r = 0.86$). **b.** Same as (a) for PT^{lower}. **c.** Number of genes detected (**Methods**), from
464 PT^{upper} neurons in bulk RNA-Seq and the union of scRNA-Seq measurements. The majority of genes
465 were detected by both methods. **d.** Same as (c) for PT^{lower}. **e.** Total genes detected by bulk RNA-Seq and

466 scRNA-Seq in PT^{upper} and PT^{lower} neurons. **f.** Number of genes detected in scRNA-Seq for each PT^{upper}
467 and PT^{lower} neuron ('X', median; PT^{upper}, 9936 genes; PT^{lower}, 9865 genes).

468 **Extended data figure 6. Differentially expressed (DE) genes in PT^{upper} and PT^{lower} cells, based on**
469 **bulk RNA-seq. a.** Genes identified as DE in scRNA-Seq, assessed in the bulk RNA-Seq data. Rows
470 represent genes, colored by differential scRNA-Seq expression in PT^{upper} (green) or PT^{lower} (brown).
471 Columns in the heat map represent transcript expression in individual replicates, 6 for each of PT^{upper} and
472 PT^{lower}. Colors show transcript intensity, as reflected from reads per kilobase of transcript per million
473 mapped reads (RPKM), shown as Z-score. Pure blue shows replicates with very low expression (Z-score
474 = -2); pure red shows replicates with very high expression (Z-score = +2). Bottom, distribution of z-
475 scores. **b.** Same plot, for genes identified as DE from bulk RNA-Seq.

476 **Extended data figure 7. Electrophysiology and trial-averaged spike rates for all identified PT**
477 **neurons. a.** PT^{upper} neurons. Left, inter-spike interval (ISI) histograms; right, trial-averaged activity on
478 lick right (*blue*) and lick left trials (*red*). Gray shaded area in ISI histograms represents the interval of -2.5
479 ms to 2.5 ms. **b.** PT^{lower} neurons. Boxed region indicates neurons recorded in the right ALM (ipsilateral to
480 injection site in medulla). All other neurons were recorded in the left ALM (contralateral to injection site).

481 **Extended data figure 8. Bursting was detected predominantly in PT^{lower} neurons.** Bursting cells (cells
482 in which greater than 10% of inter-spike intervals were less than 5 ms) were rare in the PT^{upper} population
483 (3.3%) and more common in the PT^{lower} population (18.8%; $p = 0.006$).

484 **Extended data figure 9. Preparatory activity in the late delay epoch. a.** Time course of the linear
485 combination of neuronal activity that best differentiates trial types in the 400 ms immediately prior to the
486 go cue (late coding dimension; CD_{late}) on lick right (*blue*) and lick left (*red*) trials for PT^{upper} (*top*) and
487 PT^{lower} (*bottom*) neurons. **b.** Difference in CD_{late} projections on lick right and lick left trials (selectivity) in
488 each population. Selectivity along CD_{late} is present in both populations, and persists after the go cue, but
489 does is not strongly modulated during movement initiation.

490 **Extended data figure 10. Stability of the coding dimension (CD) across time within a trial.** The
491 coding dimension is the dimension that best discriminates trial types in a given time interval. Heat maps
492 represent the correlation of the CD across all pairs of time points. **a.** In PT^{upper} neurons the coding
493 dimension remains similar to CD_{early} for all time points preceding the go cue (including the delay epoch).
494 **b.** In PT^{lower} neurons, the coding dimension in the delay epoch is largely orthogonal to the coding
495 dimension in the sample epoch. Panels (a) and (b) show that the upcoming movement direction is
496 encoded in a persistent manner in the PT^{upper} population, but not the PT^{lower} population. *Right panels:*
497 Expanded view of the change in coding dimension around the time of the go cue. An abrupt change in the
498 coding dimension occurs immediately after the go cue onset in the PT^{lower} population. A change also
499 occurs in the PT^{upper} population, but more slowly (several hundred milliseconds), largely after initiation of
500 movement.

501 **Extended data figure 11. Decoding of trial type in PT neuron types. a.** Accuracy of trial type
502 classification by single neurons in the 400 ms immediately following stimulus onset. 24.6% (15/61) of
503 PT^{upper} neurons predicted trial type with at least 70% accuracy, whereas only 4.4% (3/69) of PT^{lower}
504 neurons did so. Mean accuracy was also significantly higher in PT^{upper} neurons (PT^{upper}: $64.4 \pm 1.0\%$;
505 PT^{lower}: $58.9 \pm 0.6\%$, mean \pm s.e.; $p < 0.0001$, two-sided Mann-Whitney U-test). **b.** Cumulative distribution
506 function of the data in (a). **c.** Neurons containing the most trial-type information after stimulus onset
507 disproportionately belong to the PT^{upper} class. The 10 most discriminative neurons all belonged to the
508 PT^{upper} population. **d-f.** Same as (a-c) but decoding only based on spike rate increases above baseline.
509 Trial-type selectivity during the sample epoch in PT^{lower} neurons was predominantly characterized by a

510 modest suppression of spiking on one trial type, likely reflecting widespread lateral inhibition.
511 Disregarding spike rate changes below baseline, no PT^{lower} neurons predicted trial type with at least 70%
512 accuracy, while the same 24.6% of PT^{upper} neurons continued to do so and accounted for 20/21 of the
513 most predictive neurons (PT^{upper} : $62.7 \pm 1.1\%$; PT^{lower} : $56.7 \pm 0.4\%$, mean \pm s.e.; $p < 0.0001$, two-sided
514 Mann-Whitney U-test). As soon as the trial type is cued by the stimulus, upcoming movement direction is
515 encoded robustly in the PT^{upper} population and only minimally in PT^{lower} cells.

516 **Extended data figure 12. Movement termination signals in PT^{lower} neurons.** **a.** Selectivity along CD_{late}
517 (same as **EDFig. 9**) for PT^{upper} (green; *left*) and PT^{lower} neurons (orange; *right*) aligned to the last lick in
518 the response epoch. **b.** Selectivity along CD_{go} (same as **Fig. 6**) aligned to the last lick for each PT type. **c.**
519 Correlation of coding dimension weights at all pairs of time points after the go cue for PT^{upper} neurons
520 (*left*) and PT^{lower} neurons (*right*) using last-lick aligned spike rates. An additional transition in the
521 population dynamics accompanies the termination of movement in PT^{lower} neurons, while there is no
522 correlate of movement termination in PT^{upper} neurons. The change in dynamics at the offset of movement
523 was somewhat less abrupt than at movement onset, likely a result of aligning data to the last lick port
524 contact, which does not precisely mark the cessation of movement. **d.** Spike raster plots (*top*) and trial-
525 averaged activity (*bottom*) for four example PT^{lower} neurons aligned to the go cue (lick right: *blue*; lick
526 left: *red*) and the last lick port contact (lick right: *dark blue*; lick left: *dark red*).

527

528 **EXTENDED DATA TABLES**

529 **Extended data table 1. Digital object identifiers for reconstructed PT neurons.**

Type	Neuron ID	DOI	Complete?
Thalamus-projecting (PT ^{upper})	AA0011	https://doi.org/10.25378/janelia.5521615	Y
Thalamus-projecting (PT ^{upper})	AA0114	https://doi.org/10.25378/janelia.5526721	Y
Thalamus-projecting (PT ^{upper})	AA0115	https://doi.org/10.25378/janelia.5526724	Y
Thalamus-projecting (PT ^{upper})	AA0122	https://doi.org/10.25378/janelia.5527240	Y
Thalamus-projecting (PT ^{upper})	AA0181	https://doi.org/10.25378/janelia.5527444	Y
Thalamus-projecting (PT ^{upper})	AA0182	https://doi.org/10.25378/janelia.5527447	Y
Thalamus-projecting (PT ^{upper})	AA0245	https://doi.org/10.25378/janelia.5527657	Y
Thalamus-projecting (PT ^{upper})	AA0261	https://doi.org/10.25378/janelia.5527717	Y
Medulla-projecting (PT ^{lower})	AA0012	https://doi.org/10.25378/janelia.5521618	Y
Medulla-projecting (PT ^{lower})	AA0133	https://doi.org/10.25378/janelia.5527273	Y
Medulla-projecting (PT ^{lower})	AA0179	https://doi.org/10.25378/janelia.5527438	Y
Medulla-projecting (PT ^{lower})	AA0180	https://doi.org/10.25378/janelia.5527441	Y
Thalamus-projecting (PT ^{upper})	AA0131	https://doi.org/10.25378/janelia.5527267	N
Thalamus-projecting (PT ^{upper})	AA0134	https://doi.org/10.25378/janelia.5527276	N
Thalamus-projecting (PT ^{upper})	AA0169	https://doi.org/10.25378/janelia.5527408	N
Medulla-projecting (PT ^{lower})	AA0132	https://doi.org/10.25378/janelia.5527270	N
Medulla-projecting (PT ^{lower})	AA0135	https://doi.org/10.25378/janelia.5527279	N
Medulla-projecting (PT ^{lower})	AA0250	https://doi.org/10.25378/janelia.5527678	N

530

531

532 **Extended data table 2. Experiments**

Figure panel(s)	Animals (group #)	Viruses injections (#)	Notes
Fig. 2 c,d and EDFig. 4	1	1,2,3,4	Retrograde nuclear labeling from medulla, thal., SC, pons
EDFig. 3	2	5,6	Retrograde labeling from medulla and thal.
Fig. 3b,c	3	7,8	Retrograde labeling from medulla and thal.
Fig 4-6, EDFig. 3 and 7-12	4,5	9,10,11	Cell-type specific electrophysiology
Fig. 6	6	N/A	Reaction time measurement
Fig. 2a-b, EDFig. 2	7	12,13	Axonal reconstruction
EDFig. 5,6	8	14,15	Retrograde labeling from medulla and thal. For bulk RNA-Seq

533

534

535 **Extended data table 3. Animals used in experiments**

Group number	Genotype	Source	Catalog #	Cohort
1	C57BL/6	Charles River	027	2F
2	EMX1-IRES-Cre	JAX	005628	3M
3	EMX1-IRES-Cre	JAX	005628	4M
4	C57BL/6J	JAX	000664	3M
5	EMX1-IRES-Cre	JAX	005628	7M, 2F
6	EMX1-IRES-Cre	JAX	005628	4M
7	C57BL/6	Charles River	027	5F
8	C57BL/6	Charles River	027	7F

536

537

538 **Extended data table 4. Virus injections**

Injection number	Injection name	Virus name	Source	Plasmid Catalog #	Titer (GC/mL)	Coordinate	Volume (nL)
1	Medulla tdT (nuclear)	AAV2-retro-CAG-H2B-tdTomato	JRC		1.3e13	M/L 1.25 (left), A/P -6.6 (bregma), D/V 4.1 and 4.5	40
2	Thalamus GFP (nuclear)	AAV2-retro-CAG-H2B-GFP	JRC		1.2e13	M/L 1.0 (right), A/P -1.5 (bregma), D/V 3.2	40
3	SC tdT (nuclear)	AAV2-retro-CAG-H2B-tdTomato	JRC		1.3e13	M/L 1.1 (right), A/P +0.3 (lambda), D/V 2.2	40
4	Pons FLAG (nuclear)	AAV2-retro-CAG-H2B-mRuby2-smFP FLAG	JRC		6.7e12	M/L 0.4 (right), A/P +0.1 (lambda), D/V 5.5	40
5	Medulla tdT	AAV2-retro-CAG-tdTomato	JRC		5.5e12	M/L 1.25 (left), A/P -6.6 (bregma), D/V 4.1 and 4.5	20 (x2)
6	Thalamus GFP	AAV2-retro-CAG-GFP	JRC	Addgene# 28014	1.3e13	M/L 1.0 (right), A/P -1.5 (bregma), D/V 3.2	30
7	Medulla FLAG	AAV2-retro-CAG-mRuby2-smFP FLAG	JRC	Addgene# 59760	9.5e12	M/L 1.25 (left), A/P -6.6 (bregma), D/V 4.1 and 4.5	20 (x2)
8	Thalamus FLAG	AAV2-retro-CAG-mRuby2-smFP FLAG	JRC	Addgene# 59760	9.5e12	M/L 1.0 (right), A/P -1.5 (bregma), D/V 3.2	40
9	Medulla Chr2	AAV2-retro-Syn-ChR2(H134R)-GFP	JRC		2.0e13	M/L 1.25 (left), A/P -6.6 (bregma), D/V 4.3	200
10	Medulla Flex Chr2	AAV-SL1-Syn-Flex-ChR2-YFP	JRC		1.9e13	M/L 1.25 (left), A/P -6.6 (bregma), D/V 4.3	60
11	Thalamus Flex Chr2	AAV-SL1-Syn-Flex-ChR2-YFP	JRC		1.9e13	M/L 1.0 (right), A/P -1.7 (bregma), D/V 3.2	50
12	ALM sparse tdT	AAV2/1-Syn-iCre AAV2/1-CAG flex rev-tdTomato	JRC		2.1e8 9.0e12	M/L 1.2 (left), A/P +2.3 (bregma), D/V 0.4 and 0.9	20 (x2)
13	ALM sparse GFP	AAV2/1-Syn-iCre AAV2/1-CAG flex rev-3xGFP	JRC		2.1e8 1.4e13	M/L 1.2 (left), A/P +2.3 (bregma), D/V 0.4 and 0.9	20 (x2)
14	Medulla tdT	AAV2-retro-CAG-tdTomato	JRC		5.5e12	M/L 1.25 (left), A/P -6.6 (bregma), D/V 4.1 and 4.5	20 (x2)
15	Thalamus GFP	AAV2-retro-CAG-GFP	JRC	Addgene# 28014	1.3e13	M/L 1.0 (right), A/P -1.5 (bregma), D/V 3.2	20

539

540 **METHODS**

541 **Animals**

542 Mice used for scRNA-seq experiments in visual cortex and ALM are described in Ref [22]. Mice used for
543 all other experiments are described in **EDTable 2** and **EDTable 3**. Mice were housed on a 12-hour light-
544 dark cycle with ad libitum access to food and water, except during behavior (described in *Mouse*
545 *behavior*).

546 **Surgical procedures**

547 All procedures were in accordance with protocols approved by the Janelia Research Campus Institutional
548 Animal Care and Use Committee and Institutional Biosafety Committee. Mice were given buprenorphine
549 HCl (0.1 mg/kg; Bedford Laboratories) and ketoprofen (5 mg/kg; Fort Dodge Animal Health) for post-
550 operative analgesia and to reduce inflammation. Surgical procedures were carried out under 1-2%
551 isoflurane anesthesia. Mice were placed in a stereotaxic headholder on a thermal blanket and their eyes
552 covered with artificial tears (Rugby). Marcaine (0.05 mL, 0.5%) was injected under the skin covering the
553 skull to be thinned. The periosteum was removed and the skull thinned overlying the sites of viral
554 injection(s). For all injections, virus was injected using a manual volume displacement injector (MMO-
555 220A, Narishige) connected to a glass pipette (5-000-2005, Drummond Scientific) pulled to a 30 μ m tip
556 (P-2000, Sutter Instruments) that was beveled to a sharp tip. Pipettes were back-filled with mineral oil and
557 virus was front-loaded prior to injection. Pipettes were inserted through the thinned bone to the
558 appropriate depth and virus injected at a rate of 10 nL/min. For electrophysiology, a fiber optic cannula
559 (CFML12L05; Thorlabs) was implanted 200 μ m above the virus injection target and a headbar implanted
560 caudal to Bregma. Dental acrylic (Jet repair; Pearson Dental) was used to secure the optic fiber and
561 headbar to the skull and protect exposed bone.

562 **Viral expression**

563 All viruses used in these experiments were adeno-associated virus (AAV) produced in the Janelia
564 Research Campus (JRC) Virus Shared Resource. Viruses used for scRNA-Seq experiments are described
565 in Ref [22]. For all other experiments, viruses incorporated the AAV2-retro capsid²⁸, with the exception
566 of axonal reconstruction experiments (**Fig. 2a,b** and **EDFig. 2**), which used AAV2 serotype 1. Viruses
567 used, viral titers, injection volumes, injection coordinates, and associated experiments are described in
568 **EDTable 2** and **EDTable 4**.

569 **scRNA-Seq**

570 Single-cell RNA-Seq data (**Fig. 1**, **Fig. 2e,f**, **EDFig. 1**, and **EFig. 5**), was collected and analyzed
571 according to Ref [22]. A total of 9,035 scRNA-Seq transcriptomes were measured from ALM neurons
572 and 12,714 from V1 neurons. To collect individual cells, we used layer-enriching dissections from brains
573 of *pan*-neuronal, *pan*-excitatory or *pan*-inhibitory recombinase lines crossed to recombinase reporters
574 (4,506 cells). This dataset was supplemented by 3,196 cells isolated from other recombinase lines.
575 Dissections without layer enrichment, or multiple layers combined, were employed for lines with sparse
576 labeling. Additional recombinase lines were selected to capture cellular diversity that was suggested by
577 ongoing analysis of the data. 1,333 additional cells were derived from viral retrograde labeling, with the
578 goal to establish correspondence between transcriptomic types and projection properties. PT neurons were
579 harvested from retrogradely-labeled brains (n=63 medulla injection; n=94 thalamus; n=100 pons; n=92
580 superior colliculus; n=2 amygdala; n=2 zona incerta; 5 retrosplenial cortex) and recombinase crosses

581 (n=10). In all cases, L5 was micro-dissected to isolate PT neurons from, for example, thalamus-labeled
582 L6 CT neurons.

583 **scRNA-Seq analysis**

584 Transcriptomic features, extracted by weighted gene co-expression network analysis²³, were clustered in
585 an iterative and bootstrapped manner. The output of this procedure is a co-clustering matrix, which shows
586 the frequency with which any cell clusters with any other cell in 100 bootstrapped iterative clustering
587 rounds. The transcriptomic clusters (i.e. putative cell types) are defined by 'cutting' the co-clustering
588 matrix to derive membership of each cell to a cluster. Each cell's membership is tested post-clustering by
589 classification algorithms to assign core vs. intermediate identity to cells⁴⁴. Cells that are reliably assigned
590 to only one cluster are called 'core' cells (19,195), cells that get assigned to more than one cluster
591 (typically two), are 'intermediate' cells (2,554 cells). Each cluster was named based on known or newly
592 discovered differentially expressed markers. ALM scRNA-Seq transcriptomes clustered into 21
593 glutamatergic, 49 GABAergic and 14 non-neuronal types. Cells labeled retrogradely from thalamus,
594 medulla, superior colliculus or pons were submitted to scRNA-Seq, with the results mapping onto the pre-
595 established taxonomy.

596 **Single-cell axonal reconstruction**

597 The axons of single neurons were labeled and imaged as described (**Fig. 2a,b** and **EDFig. 2**)²⁷. Neurons in
598 motor cortex were sparsely labeled using a viral vector encoding either eGFP or tdTomato. At least 3
599 weeks following virus injection, mice were perfused and brains extracted. Brains were embedded in
600 gelatin and cleared using a combination of DMSO and D-sorbitol. The full volume of each brain was
601 imaged at submicron resolution using an automated block-face two-photon microscope with integrated
602 vibratome. Full-brain datasets were approximately 20-30 TB in size and were stitched and rendered using
603 a custom bioinformatics pipeline²⁷. Each individual neuron was reconstructed manually in three-
604 dimensions using the Janelia Workstation⁴⁵ by two independent annotators who were blinded to all
605 analyses. Consensus reconstructions were determined by resolving discrepancies (generally <5%)
606 between annotators. Each dataset was registered to the Allen Common Coordinate Framework using
607 landmark based registration (3DSlicer, Landmark Registration module) and a thin plate spline warp
608 determined between the two image spaces. Neuronal reconstructions were then projected into the
609 reference space to determine the brain area associated with each axonal segment, branch point, and
610 terminus.

611 **Histology and imaging**

612 At least three weeks following viral injections, mice were trans-cardially perfused with PBS (>20 mL)
613 followed by 4% paraformaldehyde (>20 mL). Brains were post-fixed overnight. For immunolabeling
614 (**Fig. 2c,d** and **EDFig. 4**), brains were transferred to a 20% sucrose solution for cryoprotection and
615 sectioned coronally at 50 μ m on a freezing microtome. In all other cases (**EDFig. 3**), brains were
616 sectioned at 50 or 100 μ m on a vibratome (VT1200; Leica Biosystems). Sections were processed using
617 standard immunohistochemical techniques and imaged as described previously (**Fig. 2c,d** and **EDFigs.**
618 **3,4**)⁴⁶. Brightness and contrast were adjusted manually to match approximate luminance values across
619 imaging experiments in a linear fashion using ImageJ/Fiji.

620 **Bulk RNA-Seq**

621 Cells back-labeled from thalamus, medulla, pons and superior colliculus (SC) with GFP or tdTomato
622 were collected by manual cell sorting, as described (**EDFig. 5** and **EDFig. 6**)⁴⁷. 50-70 cells were collected
623 per sample, with 7 experimental replicates performed for each. Cells were isolated by manual cell sorting

624 on a fluorescence dissecting scope, following micro-dissection, trituration and enzymatic digestion⁴⁸.
625 Following pooling and lysis, total RNA was extracted by Picopure kit (KIT0204; Thermo-Fisher).
626 Amplified DNA was produced using Ovation RNA-Seq System V2 kit (#7102; NuGEN), fragmented to
627 ~200bp, ligated to Illumina sequencing adaptors with Encore Rapid kit (#0314; NuGEN), and sequenced
628 on an Illumina HiSeq 2500 with 4-fold multiplexing (single end, 100bp read length).

629 **Bulk RNA-Seq analysis**

630 Adaptor sequences (AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC) were removed from reads
631 using Trimmomatic 0.36⁴⁹, mapped using STAR 2.5.3a⁵⁰ to the Ensembl mouse genome GRCm38.p5,
632 release 90 (<https://www.ensembl.org>). Mapped reads were normalized to the total number of reads per
633 sample (counts per million). Differential-expression (DE) criteria were: false-discovery-rate < 5%; log₂-
634 fold-change > 2.0; sample CPM mean > 10.0 in 3 or more replicates. Principal components analysis of the
635 7 thalamus-projecting and 7 medulla-projecting replicates showed that a single replicate from each
636 behaved as an outlier. Thus, for further analysis, 6 replicates were considered for each. For selection of
637 potential marker genes, non-coding RNAs and gene models were removed from the DE gene lists. For
638 visualization, reads per kilobase of transcript per million mapped reads (RPKM) were used.

639 Similar numbers of genes were detected in each of the thalamus and medulla bulk RNA-Seq samples
640 (replicate-averaged #detected genes: 12,317 and 12,363 respectively). These numbers were slightly higher
641 than the genes detected from scRNA-Seq (thalamus: 11,435; medulla: 11,573; pons: 9,486; and SC:
642 9,742, respectively).

643 ***In situ* hybridization**

644 Animals were perfused with 4% PFA and brains were post fixed in 4% PFA overnight at 4 degrees C.
645 Brains were then rinsed in PBS and cryoprotected in 30% sucrose, and 20 μm thick sections were cut on a
646 cryostat. smFISH followed by IHC was performed on fixed frozen tissue from mice injected with AAV2-
647 retro-mRuby2-smFP-FLAG either in thalamus or in medulla (**EDTables 2-4**) as per protocols for fixed
648 frozen tissue using proprietary probes from Advanced Cell Diagnostics (ACDBio). Probes used in this
649 study (Mm-Npnt: Cat# 316771; Mm-Slco2a1: Cat# 485041) were detected using propriety detection
650 reagent (RNAscope Fluorescent Multiplex Detection Reagents Cat # 320851), using Amp4 Alt B (Atto
651 550). Following smFISH, sections were rinsed in PBS, and blocking buffer (2% BSA and 0.3% serum)
652 was applied for 5 minutes. Primary antibody (Sigma-Millipore F1804) was diluted in the blocking buffer
653 (1:100) and incubated overnight at 4 degrees C. Sections were rinsed in PBS 3 times (5 minutes) and
654 incubated with secondary antibody Goat anti-mouse AF 488 (A-11001, Invitrogen; diluted 1:100 in
655 blocking buffer) at room temperature for 2 hrs. Sections were then rinsed in PBS and cover slipped with
656 Vectashield containing DAPI (H-1500; VectorLabs). Images for display were acquired as a single plane
657 on a Zeiss 880 inverted confocal microscope, using a 63X/1.4NA objective. Images for quantification
658 were acquired on 7μm thick stacks using a 40X oil immersion objective/1.3NA (pixel size, 0.11 X 0.11
659 μm). Laser power was adjusted across sections to achieve maximum dynamic range. Punctate mRNA
660 signal was quantified on cell volumes from maximum intensity projection of the Z-stacks. Brightness and
661 contrast were adjusted manually to match approximate luminance values across imaging experiments in a
662 linear fashion using ImageJ/Fiji.

663 **Mouse behavior**

664 Mice were water restricted and housed on a 12-hour reverse light-dark cycle with testing during the dark
665 phase. On days in which mice were not trained, they received 1 mL of water. Behavioral experiments
666 lasted one to two hours per day, during which period they consumed their daily water intake (~ 0.5 to 1.0

667 mL). Mice unable to sustain stable body weight were given supplementary water. Mice were trained using
668 operant conditioning as previously described^{26,51} until reaching behavioral criterion (>75% trials correct).
669 At the beginning of each trial, a vertical pole moved into place adjacent to the whisker pad and in reach of
670 the whiskers (200 ms travel time). The pole remained in this position for 1.0 sec and then was retracted
671 (200 ms travel time). The sample epoch was defined as the 1.0 sec during which the pole was in range of
672 the whiskers and stationary. After the pole was removed, the mouse was trained to refrain from licking for
673 an additional 1.3 sec (delay epoch) before an auditory ‘go cue’ (pure tone, 3.4 kHz, 0.1 s duration)
674 instructed the mouse to lick (reward epoch). Premature licks during the sample or delay epoch resulted in
675 a restart of the requisite epoch and these trials were excluded from all analyses. Licking the correct
676 lickport after the go cue led to a small water reward (3 μ L). Licking the incorrect lickport triggered a
677 timeout (2-10 sec). Trials in which mice did not lick within a 1.5 sec window after the go cue were rare
678 and typically occurred at the end of a session.

679 **Videography**

680 High-speed video was acquired at 400 Hz from below and to the side of the mouse using CCD cameras
681 (CM3-U3-13Y3M; FLIR) with a 4-12mm focal length lens (12VM412ASIR; Tamron). Camera data were
682 acquired using BIAS (IORodeo). Reaction times were determined by measuring the luminance change in
683 a small ROI manually placed just below the jaw in side-view movies. Luminance traces were averaged
684 across all of the trials within each session (453 ± 79 mean \pm s.d.; range: 295 - 551). 95% confidence
685 intervals for reaction time were calculated by bootstrapping session means across mice ($n = 3$) and
686 sessions ($n = 14$). Little inter-animal variation was observed in session-averaged reaction time.

687 **Electrophysiology**

688 A small craniotomy (diameter, 0.5 - 1 mm) was made over ALM one day prior to the first recording
689 session. Extracellular spikes were recorded using silicon probes containing two shanks each with 32
690 channels with 25 μ m spacing (H2; Cambridge Neurotech). The 64 channel voltage signals were
691 multiplexed, recorded on a PCI6133 board (National instrument), and digitized at 14 bits. The signals
692 were demultiplexed into the 32 voltage traces, sampled at 25 kHz and stored for offline analyses. 4-7
693 recordings were made from each craniotomy on consecutive days. Recording depth was inferred from
694 manipulator readings without compensation for cortical curvature. The tissue was allowed to settle for 10
695 minutes prior to recording.

696 To optogenetically tag PT^{upper} and PT^{lower} neurons during recording, we expressed ChR2(H134R)-YFP
697 selectively in each population using a viral injection into the medulla or thalamus as described above.
698 During each recording session, >1200 optical stimuli were delivered at 4 Hz just prior to and following
699 the behavioral session. Stimuli were 0.1-0.5 ms at 80-100 mW (measured just before the implanted fiber
700 optic cannula). Reliable antidromic activation was observed in 1-6 units per session. Due to the proximity
701 of the cerebral peduncle, subthalamic nucleus, and zona incerta, which contain projections from both cell
702 types, a smaller viral injection was performed in the thalamus (50 nL) than in the medulla (50-200 nL),
703 resulting in fewer tagged PT^{upper} neurons and reduced throughput. Across all sessions recording PT^{upper}
704 neurons, mouse performance was $85.4 \pm 7.8\%$ on lick left trials and $89.3 \pm 9.4\%$ on lick right trials (mean
705 \pm s.d.; $n = 37$ sessions in 8 mice; left hemisphere). In PT^{lower} neuron recordings, performance was $83.5 \pm$
706 6.3% on lick left trials and $86.3 \pm 8.2\%$ on lick right trials ($n = 23$ sessions in 4 mice; both hemispheres).
707 Mice performed a median of 107 and 105 correct trials on lick left and lick right trials respectively in
708 PT^{upper} recordings and 117 and 108 correct trials during PT^{lower} recordings. PT neurons recorded from the
709 left and right ALM did not differ qualitatively and were combined in all further analysis to increase
710 statistical power.

711

712 Electrophysiology data analysis

713 Extracellular recorded traces were band-pass filtered (300 – 4500 Hz; 2nd order Butterworth filter) and the
 714 common mode on ± 4 sites was subtracted from each channel. Events were detected using JRClust⁵² and
 715 spikes from tagged units ($n = 143$) were sorted manually using a custom program written in MATLAB.
 716 Extreme care was taken to restrict analysis to units that could be sorted with a low number of false
 717 positive spikes (mean ISIs less than 2 ms = 0.02%; **EDFig. 7**) so that neuronal responses could be
 718 faithfully attributed to the correct cell type. Despite this, spike rates were similar to that measured in other
 719 studies recording extracellular from ALM in the same task¹⁸ indicating that the false negative spike rate
 720 remained low. Information from 4-7 adjacent used for sorting each individual unit. Units recorded during
 721 behavioral sessions in which performance was not greater than 65% for both trial types were excluded
 722 from the dataset. Additionally, units recorded during behavioral sessions with less than 50 correct trials of
 723 each type were excluded. 13 units were rejected based on these criteria and 130 were kept for further
 724 analysis. Unit depths (**Fig. 4e**) were inferred from manipulator readings only without correction for the
 725 angle between the electrode penetration and the orientation of cortical layers. Collision tests were
 726 performed for all tagged units to confirm axonal projections to the thalamus or medulla^{18,53} (**Fig. 4d**).
 727 Trial-averaged spike rates were calculated in 5 ms time bins and filtered using a causal 50 ms boxcar
 728 filter.

729 Coding dimension vectors (**Figs. 5,6** and **EDFigs. 9,12**), **CD**, were calculated according to equations 1
 730 and 2.

$$731 \quad \mathbf{v} = \frac{\bar{x}_{lick\ right} - \bar{x}_{lick\ left}}{\sqrt{Var(\bar{x}_{lick\ right}) + Var(\bar{x}_{lick\ left})}} \quad (1)$$

$$732 \quad \mathbf{CD} = \frac{\mathbf{v}}{\sum |\mathbf{v}|} \quad (2)$$

733 For each unit, the mean difference in spike rate between lick right, $\bar{x}_{lick\ right}$, and lick left trials,
 734 $\bar{x}_{lick\ left}$, was calculated across a 400 ms time interval. This vector was divided by the square root of the
 735 sum of the across-trial variances of spike rate for each trial type. The resulting vector (\mathbf{v}) was then
 736 normalized by its L1 (taxicab) norm so that projections would not scale with vector length (# cells in
 737 population) to produce the coding dimension, **CD**. Coding dimensions were calculated in the first 400 ms
 738 of the sample epoch (CD_{early} ; 2.5 to 2.1 seconds before the go cue) the last 400 ms of the delay epoch
 739 (CD_{late} ; 0.4 to 0.0 seconds before the go cue) and the first 400 ms of the response epoch (CD_{go} ; 0.0 to 0.4
 740 seconds after the go cue). CD_{go} was further orthogonalized to CD_{late} using the Gram-Schmidt process to
 741 remove the component of CD_{late} that persisted through the response epoch from CD_{go} . In all cases, coding
 742 dimensions were calculated separately for the PT^{upper} and PT^{lower} populations.

743 Projections of the activity of each PT population along the coding dimensions ($\mathbf{p}_{lick\ right}, \mathbf{p}_{lick\ left}$) were
 744 obtained according to equations 3 and 4:

$$745 \quad \mathbf{p}_{lick\ right} = \mathbf{CD}^T \mathbf{x}_{lick\ right} \quad (3)$$

$$746 \quad \mathbf{p}_{lick\ left} = \mathbf{CD}^T \mathbf{x}_{lick\ left} \quad (4)$$

747 Selectivity, **S**, along each dimension was calculated as:

$$748 \quad \mathbf{S} = \mathbf{p}_{lick\ right} - \mathbf{p}_{lick\ left} \quad (5)$$

749 In all cases (**Figs. 5,6** and **EDFigs. 9,12**), the illustrated projections are the results of a hierarchical
750 bootstrapping procedure. For each population, 100 projections were calculated, each using N randomly
751 chosen neurons (with replacement), where N was the number of neurons in the population. For each
752 neuron, 50 correct trials of each trial type were randomly selected with replacement. In all figures, solid
753 lines and shaded areas represent the median and standard deviation of all repetitions.

754 To create matrices of the coding dimension correlation across time (**EDFig. 10**), coding dimensions were
755 calculated at each time point from the trial-averaged spike rates of all neurons within a population (5 ms
756 time bins, filtered using a causal 50 ms boxcar filter), as above, but were normalized by their Euclidean
757 norms to produce unit vectors. Correlation matrices represent the inner product of coding dimension
758 vectors at each pair of time points.

759 Single-cell trial type decoding accuracy (**EDFig. 11**) was determined using the average spike rate during
760 the first 400 ms of the sample epoch (2.5 to 2.1 seconds before the go cue). A spike rate threshold was
761 determined that best distinguished lick right trials from lick left trials (maximal accuracy) with accuracy
762 defined as the proportion of trials correctly classified. Accuracy was $\geq 50\%$ by definition. Shaded
763 regions in around the cumulative distribution function in **EDFig. 11b,e** represent standard error, estimated
764 using Greenwood's formula. In **EDFig. 11c,f**, confidence intervals represent standard errors (bootstrap)
765 and the gray shaded region represents ± 1 standard error of the expected value after shuffling cell type
766 labels (100,000 repetitions).

767 **Code availability**

768 All analysis code used in this study is available upon request.

769

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776

777 **AUTHOR CONTRIBUTIONS**

778 MNE, KS, SV, LL, BT, and HZ; conception and design of the experiments. BT, TNN, LTG and HZ;
779 scRNA-seq experiments, with material provided by MNE and SV. MNE, EB, JW, and JC; single neuron
780 reconstructions. SV; in situ experiments and analysis. MNE; electrophysiology experiments. MNE, SV,
781 and CRG; anatomical experiments. MNE, SV, VM, LG, TN, KS and LL; data analysis. MNE, KS, and
782 LL; wrote the paper with input from all authors.

783

784 **DATA AVAILABILITY**

785 Single cell transcriptomic data will be available through GEO. Bulk RNA-Seq data will be deposited in
786 the NCBI Sequence Read Archive. Electrophysiology data sets will be shared at CRCNS.ORG in the
787 NWB format.

Figure 1

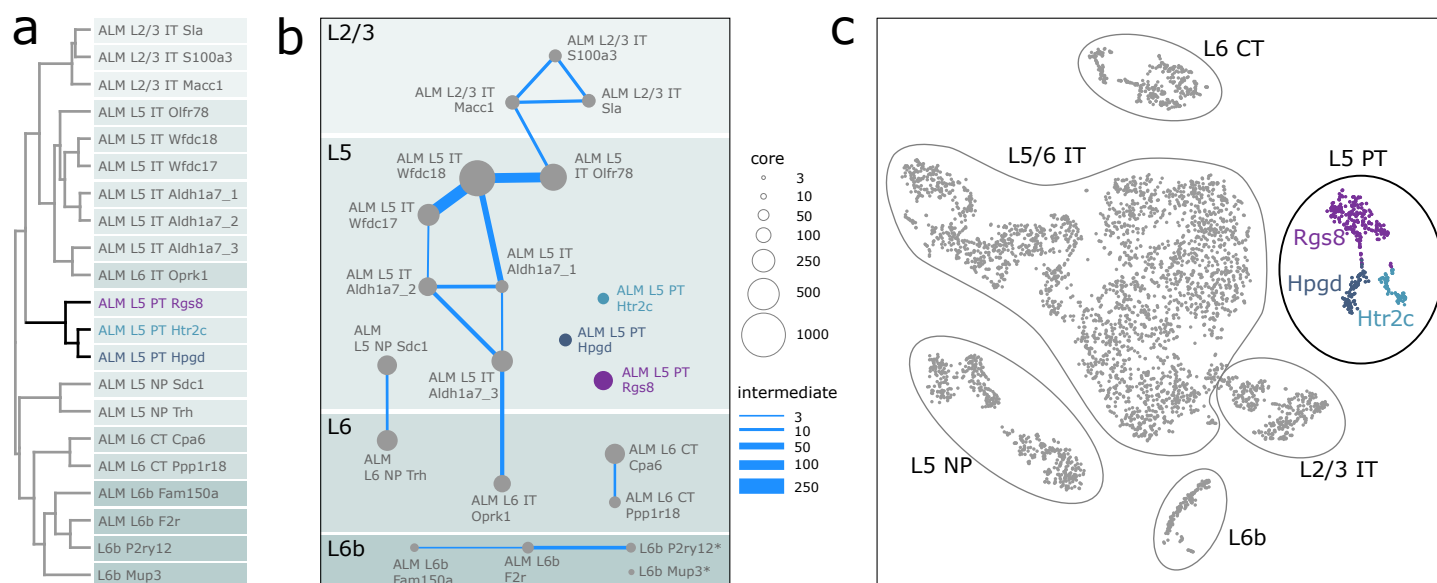


Figure 1. Taxonomy of motor cortex glutamatergic neurons based on single-cell RNA-seq. a. Hierarchical clustering of gene expression. Three gene expression clusters, identified by the genes *Rgs8*, *Htr2c*, and *Hpgd*, correspond to pyramidal tract neurons. **b.** Constellation diagram of gene expression clusters in ALM. Node diameters indicate the number of neurons belonging to each cluster (core) and edges represent cells shared by two clusters (intermediates). **c.** Two-dimensional stochastic neighbor embedding (tSNE) projection of transcriptomic data of sequenced single neurons in ALM. The cluster memberships of individual neurons are color-coded as in panels (a) and (b).

Figure 2

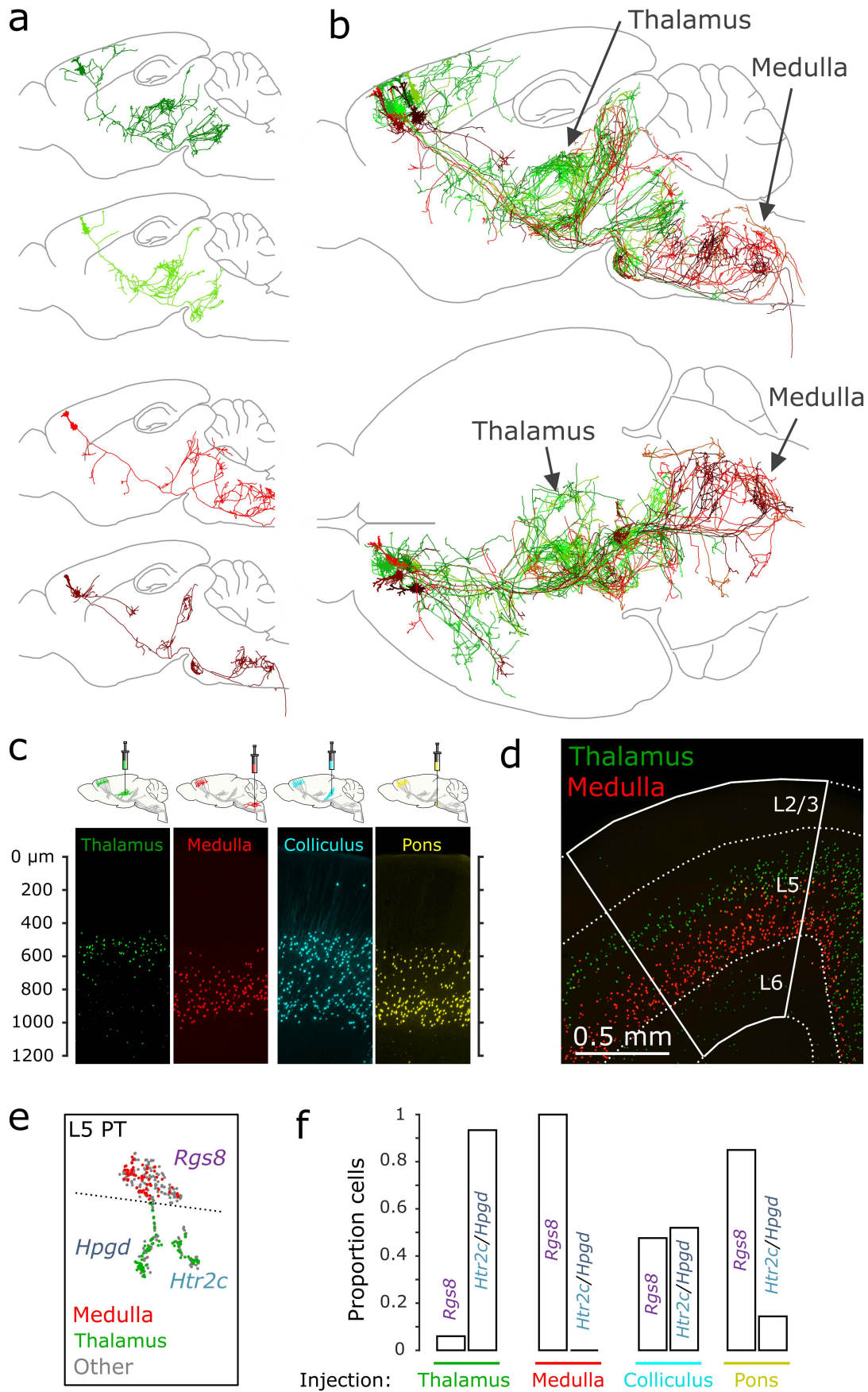


Figure 2. Two types of PT neuron in motor cortex. **a.** Example dendritic and axonal reconstructions of four single PT neurons. Two PT neurons project to the thalamus (*top; yellow-green hues*) and two project to the medulla (*bottom; red-brown hues*). **b.** Reconstructions of 4 thalamus-projecting PT neurons and 4 medulla-projecting PT neurons overlaid and collapsed in the sagittal (top) and horizontal (bottom) planes. Dendrites are denoted by thicker line segments. **c.** Nuclei of neurons retrogradely labeled from different PT targets are located in distinct sublaminae of L5b. **d.** Nuclei of PT neurons retrogradely labeled from the thalamus (*green*) or the medulla (*red*). **e.** Gene expression of PT neurons retrogradely labeled from the thalamus (*green*) and medulla (*red*) in tSNE space (as in Fig. 1c). PT neurons projecting to the medulla belong to the *Rgs8* cluster, whereas thalamus-projecting PT neurons were part of the *Htr2c* and *Hpgd* clusters. **f.** Proportion of neurons retrogradely labeled from each PT target that were clustered into the *Rgs8* and *Htr2c/Hpgd* expression clusters.

Figure 3

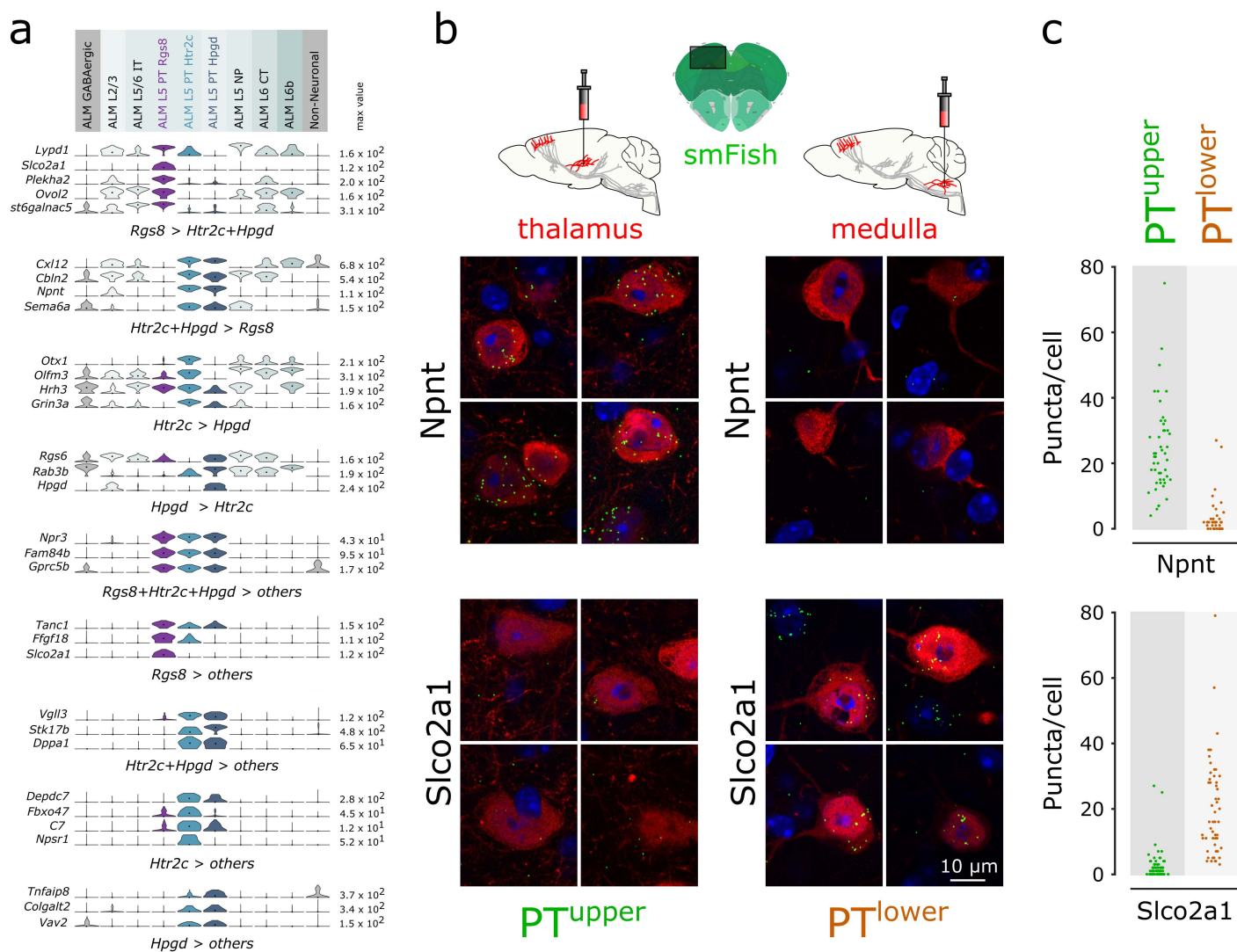


Figure 4

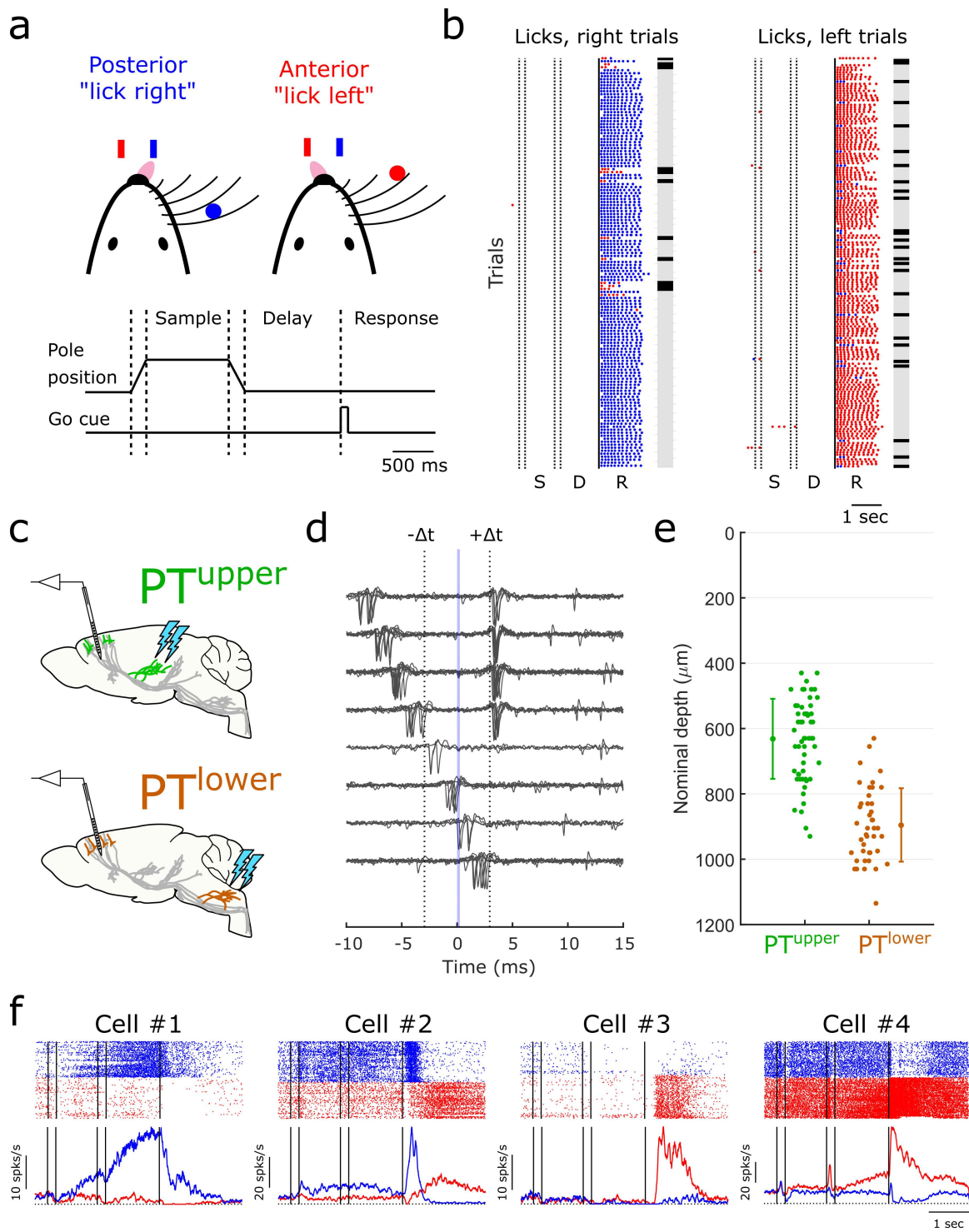


Figure 4. Cell type-specific extracellular neurophysiology. **a.** Mice were trained on a delayed-response task. On each trial, an object appeared within reach of the whiskers in one of two rostro-caudal positions during the sample epoch (1.0 s). The pole was removed and after a brief delay epoch (1.3 s), mice reported the pole position by licking a reward port on the right (caudal pole position) or the left (rostral pole position). **b.** Performance during an example session. Dots represent licks to the right (*blue*) or the left (*red*). Gray and black marks indicate correct and incorrect trials, respectively. **c.** Schematic for stimulation and recording configuration for each cell type. **d.** Collision test for an example neuron. Trials with spontaneous spikes preceding the light-evoked spike are shown binned by the latency of spikes preceding the stimulus from top to bottom. Putative photostimulation-evoked spikes are at $+\Delta t$. When a spike occurs in the interval $[-\Delta t, +\Delta t]$, a collision occurs with the photostimulation-evoked spike in the axon and the spike at $+\Delta t$ is absent. **e.** Depth distribution of PT^{upper} and PT^{lower} neurons based on micromanipulator readings. Depths are measured from the dorsal surface and are uncorrected for the curvature of cortical layers. Error bars represent mean \pm s.d. **f.** Example identified neurons. *Top*: spike rastergrams for correct lick right trials (*blue*) and lick left trials (*red*). *Bottom*: trial-averaged spike rates. S, sample; D, delay; R, response.

Figure 5

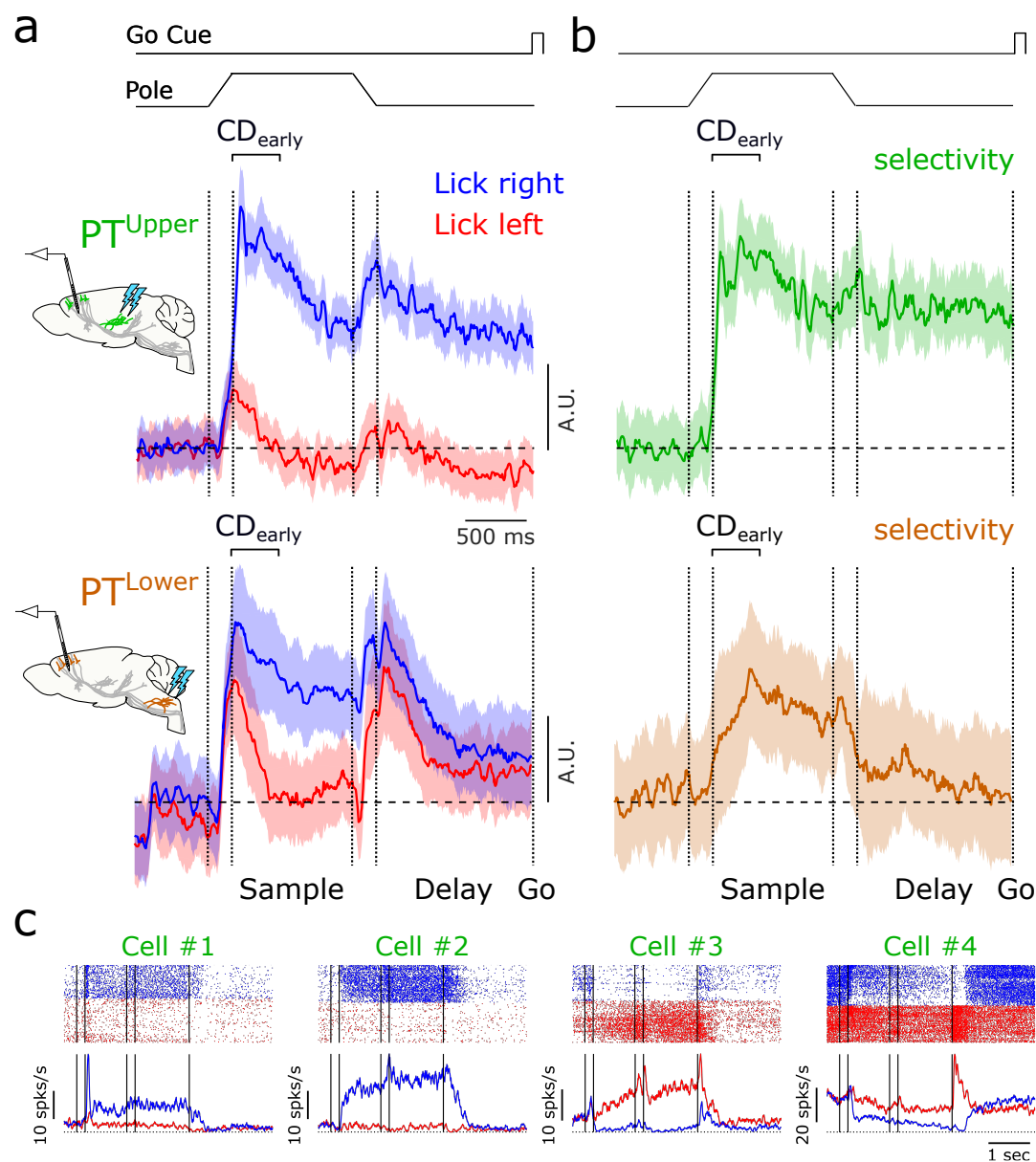


Figure 5. Persistent preparatory activity in PT^{upper} neurons. **a.** Time-course of the linear combination of neuronal activity that best differentiates trial type after stimulus onset (CD_{early}) on lick right (blue) and lick left (red) trials for PT^{upper} (top) and PT^{lower} (bottom) neurons. **b.** Difference in CD_{early} projections on lick right and lick left trials (selectivity) in PT^{upper} (top; green) and PT^{lower} (bottom; orange) neurons. **c.** Example identified PT^{upper} neurons. *Top:* raster plots for correct lick right trials (blue) and lick left trials (red). *Bottom:* trial-averaged spike rates. Shaded regions in (a) and (b) represent 95% confidence intervals around the mean using hierarchical bootstrapping.

Figure 6

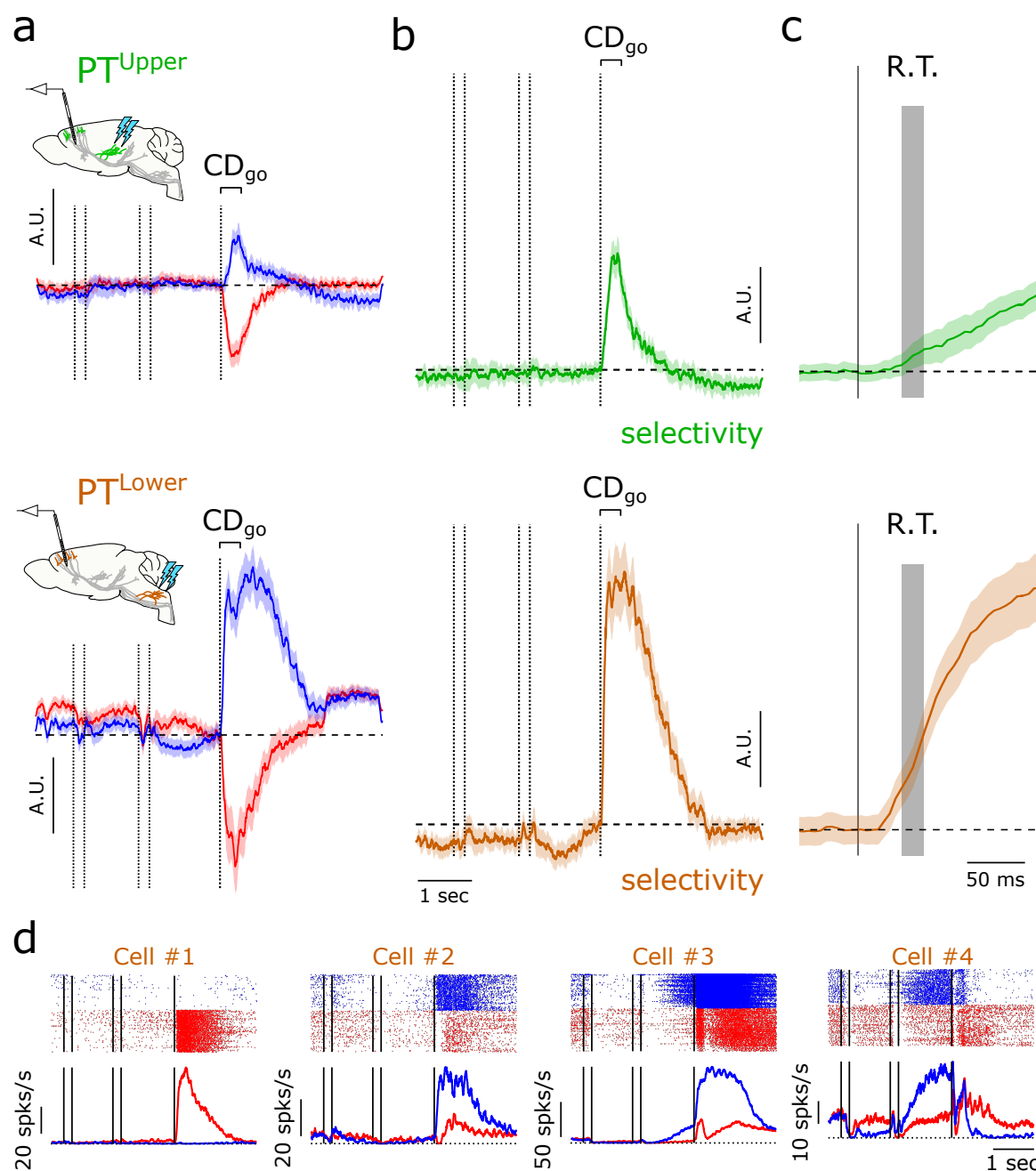
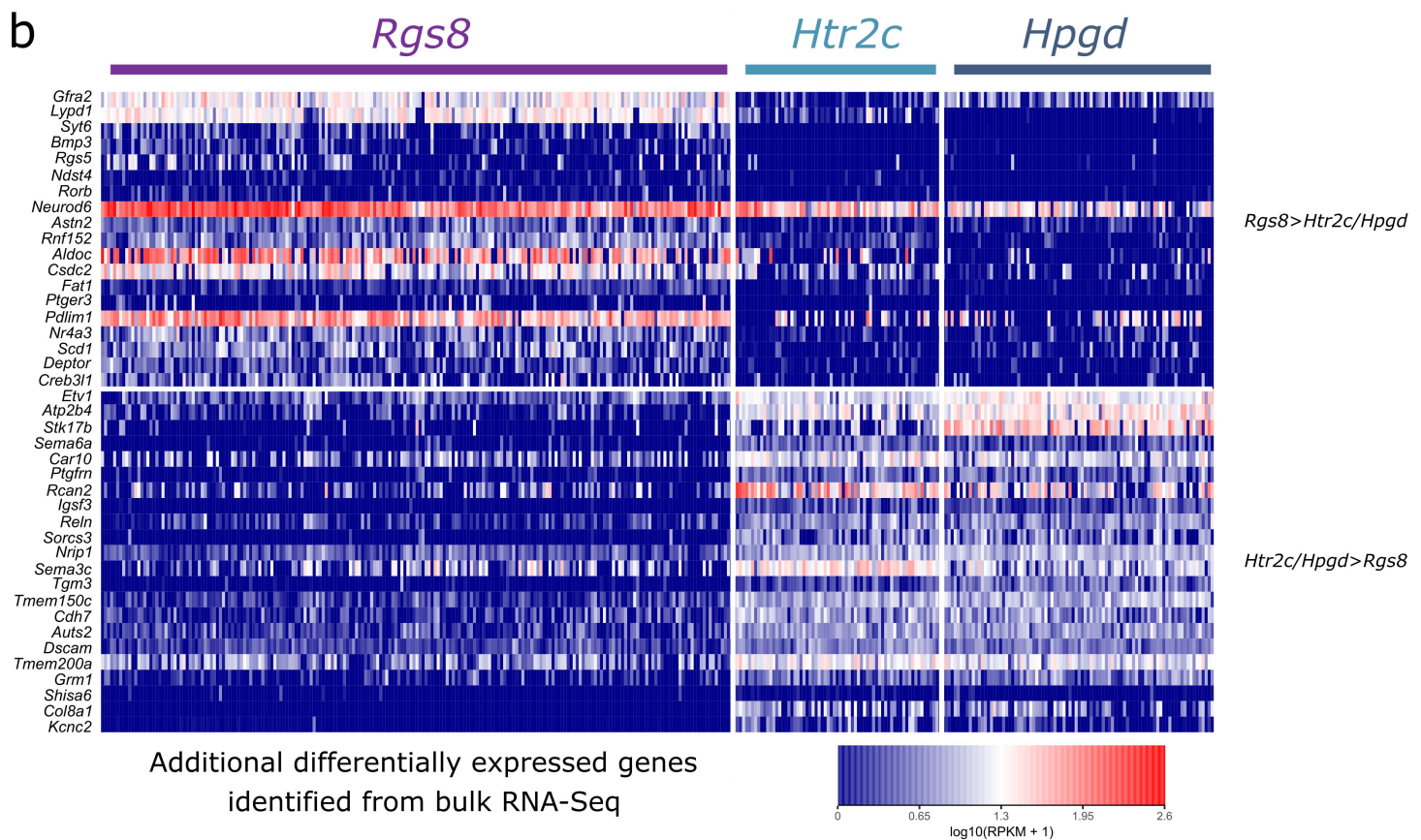
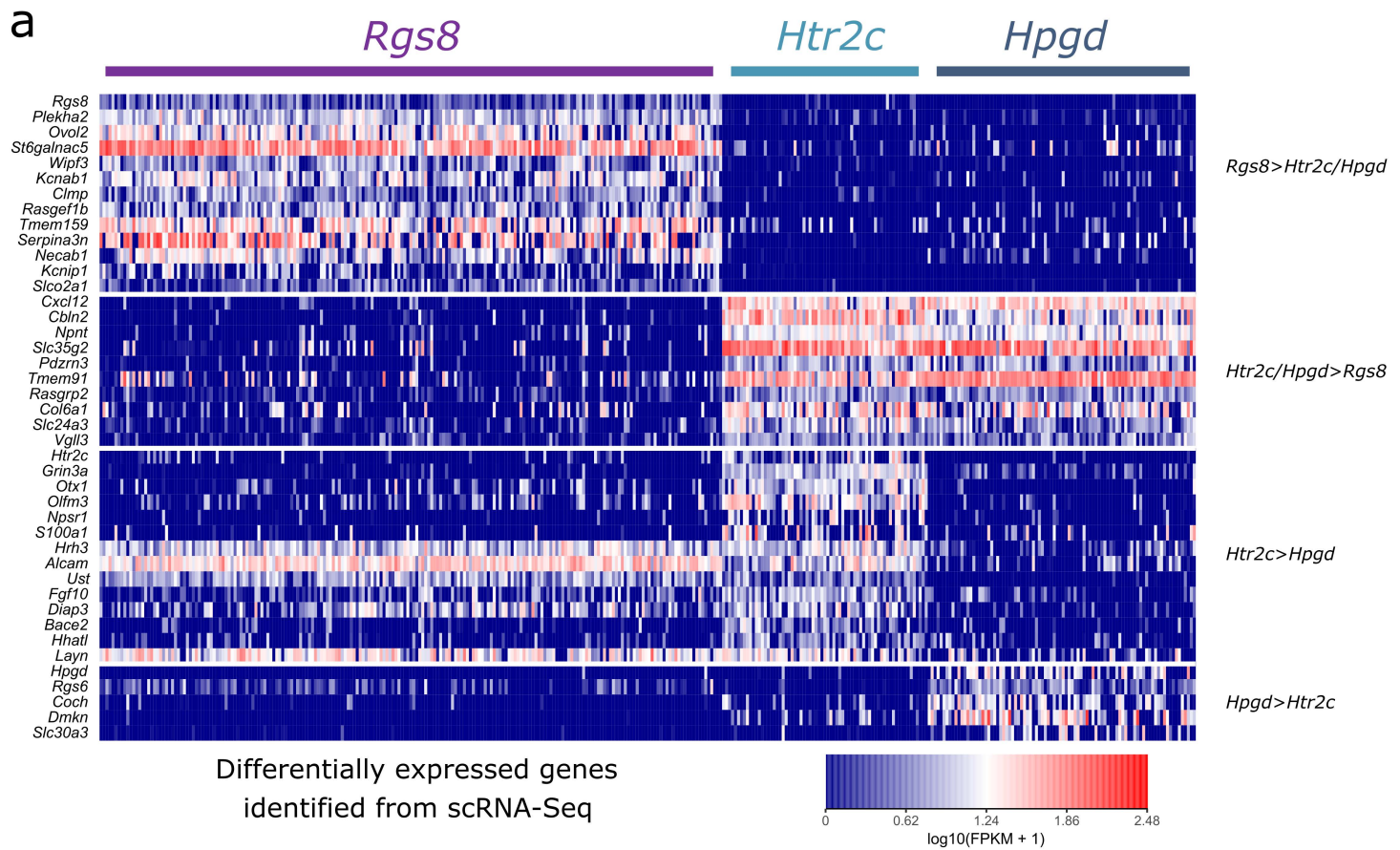


Figure 6. Movement commands in in PT^{lower} neurons. **a.** Time-course of the linear combination of neuronal activity that best differentiates trial types after the go cue (CD_{go}) on lick right (blue) and lick left (red) trials for PT^{upper} (top) and PT^{lower} (bottom) neurons. **b.** Difference in CD_{go} projections on lick right and lick left trials (selectivity) in PT^{upper} (top; green) and PT^{lower} (bottom; orange) neurons. **c.** Data from (b) expanded around the go cue. Gray region indicates the distribution of session-averaged reaction times (earliest detected orofacial movement, *R.T.*; 99% confidence interval = 38-56 ms). Along the CD_{go}, selectivity in PT^{lower} neurons emerged 24 ms following the go cue, faster than in the PT^{upper} population (46 ms) and consistent with a role in movement initiation. **d.** Example identified PT^{lower} neurons. *Top*: raster plots for correct lick right trials (blue) and lick left trials (red). *Bottom*: trial-averaged spike rates. Shaded regions in (a-c) represent 95% confidence intervals around the mean using hierarchical bootstrapping.

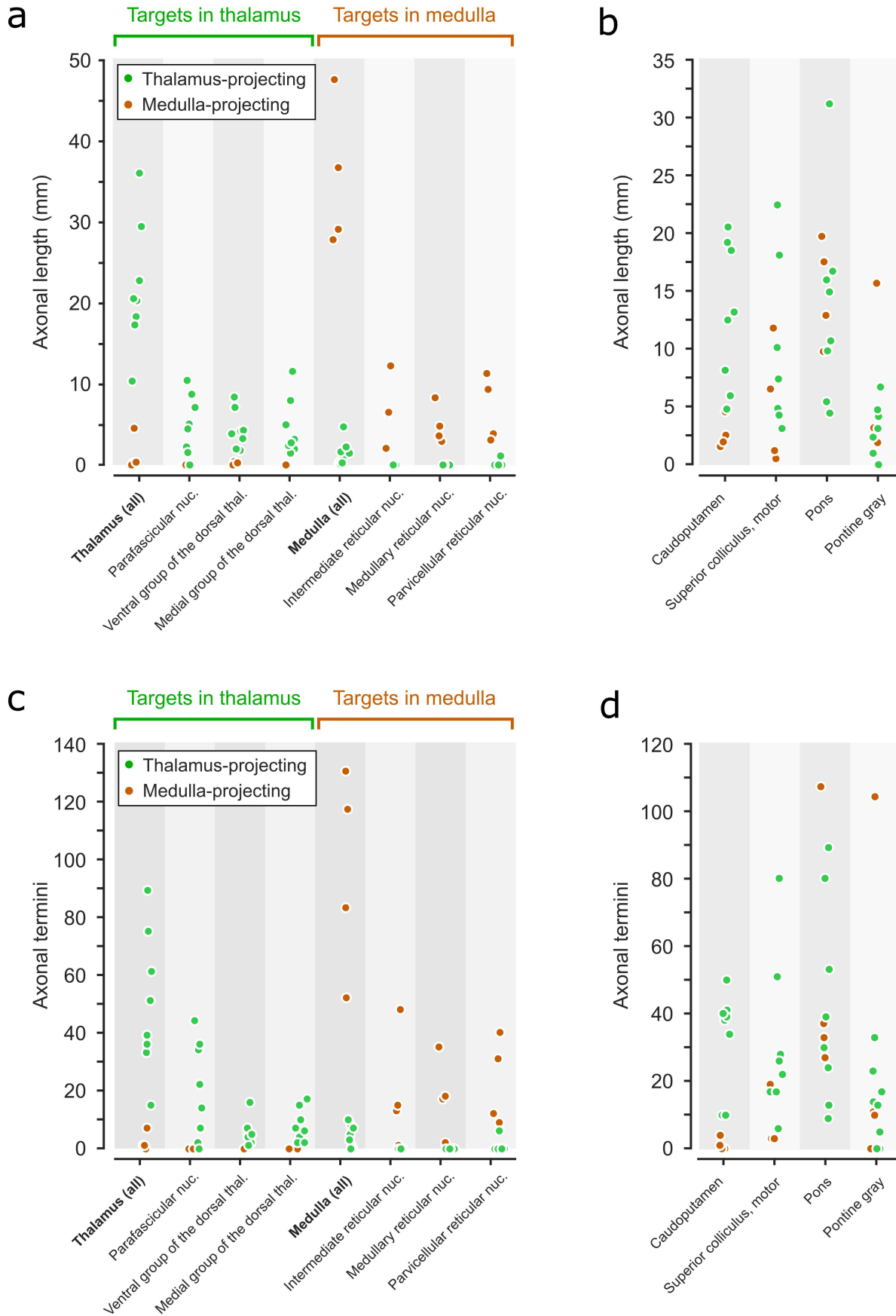
Extended data figure 1



Extended data figure 1. Differentially expressed genes in single cell RNA-seq PT neuron expression.

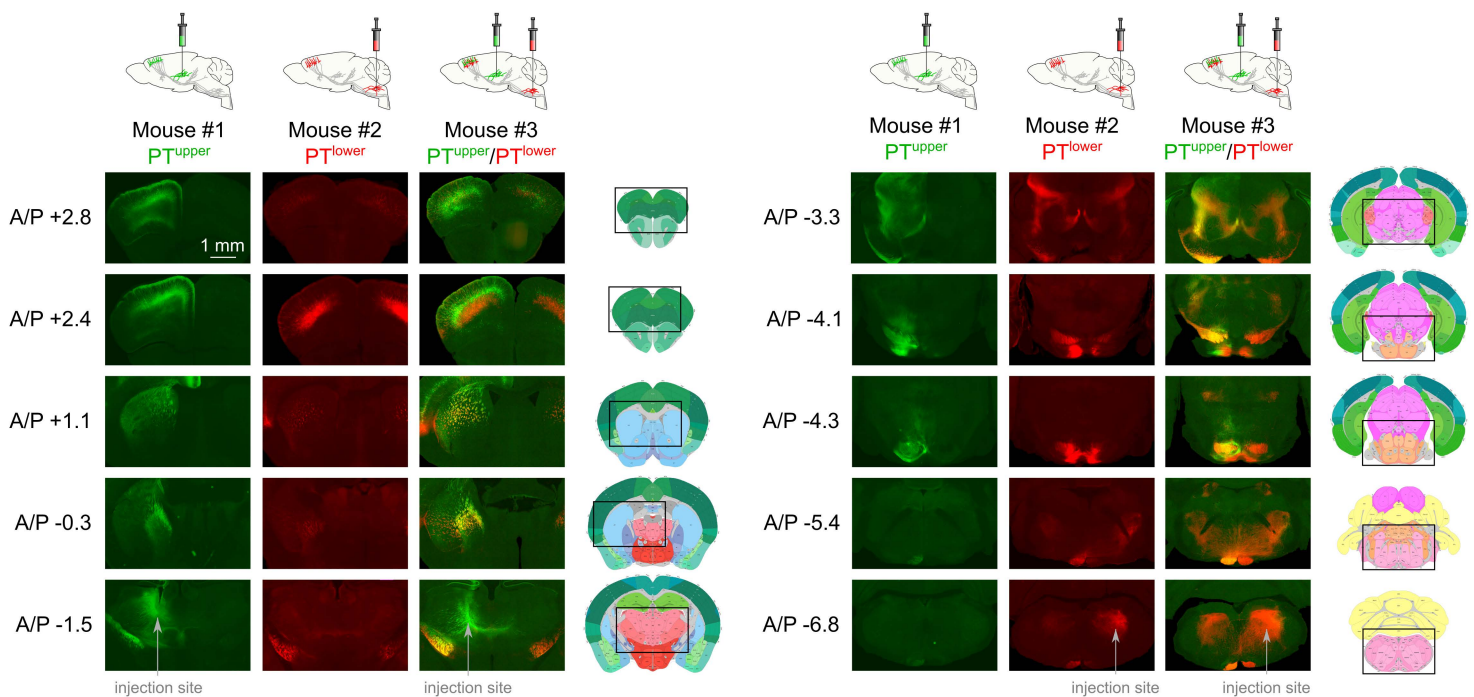
a. Heat map of expression of differentially expressed genes. Columns in the heat map represent individual cells, grouped by cluster (*Rgs8*, $n=209$; *Htr2c*, $n=69$; *Hpgd*, $n=90$). Rows represent genes selected by the clustering algorithm to represent transcriptomic branch points between clusters. Color shows transcript expression in fragments per kilobase of transcript per million mapped reads (FPKM), shown on a log-scale. Pure blue shows cells with 0 transcripts of a given gene; pure red shows cells with maximal expression (approximately 300 FPKM). **b.** Expression data for the same cells in (a) for genes identified from bulk RNA-Seq as differentially expressed between the *Rgs8* and *Hpgd/Htr2c* clusters.

Extended data figure 2



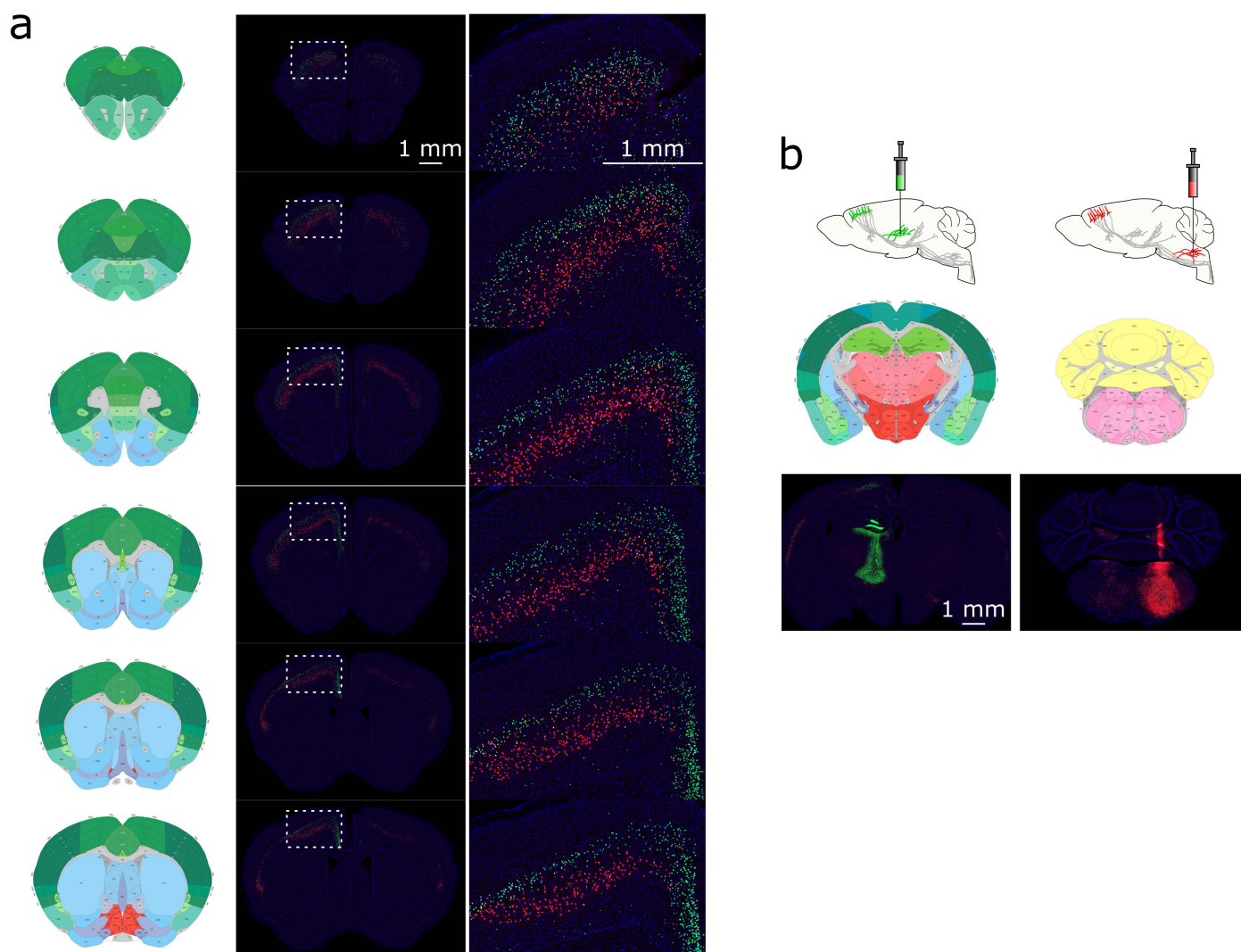
Extended data figure 2. Distribution of axonal projections. **a.** Axonal length within thalamic and medullary targets for thalamus-targeting PT neurons ($n = 8$; *green*) and medulla-targeting PT neurons ($n = 4$; *orange*). **b.** Axonal lengths within other selected PT targets. **c.** Axonal termini within thalamic and medullary targets. **d.** Axonal termini within other selected PT targets.

Extended data figure 3



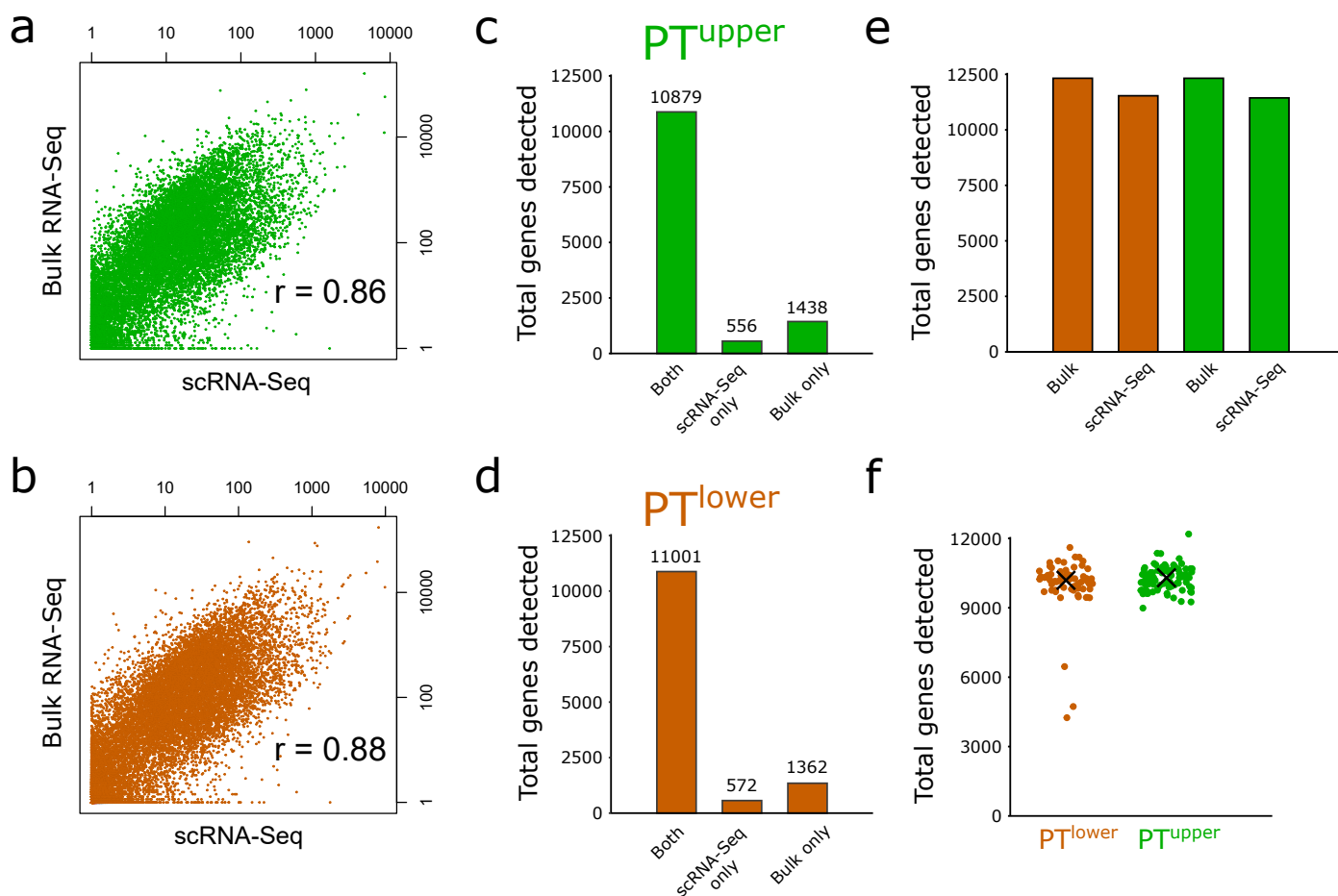
Extended data figure 3. Anterograde anatomy: targets of PT^{upper} and PT^{lower} populations. Groups of cortical neurons were labeled from the thalamus (PT^{upper} ; Mouse #1), the medulla (PT^{lower} ; Mouse #2), or both targets (Mouse #3) using AAVretro expressing spectrally-distinct fluorescent proteins. Top, schematics of the labeling procedures. Left, rostral-caudal level (relative to Bregma). Right, imaged area is indicated on annotated coronal sections taken from the Allen Reference Atlas at the corresponding rostral-caudal level. Both types extended axon collaterals to motor-related SC, but to different parts. Axons from PT^{upper} cells were apparent throughout all SC layers, with a particularly dense projection to the ventrolateral aspect, whereas PT^{lower} neurons were restricted to the ventral superior colliculus and were concentrated more caudally. Both groups projected to the pons, particularly the pontine gray, but with terminations in largely non-overlapping zones. PT^{upper} cells exclusively projected to the globus pallidus external segment and broadly targeted the dorsal, lateral and ventral striatum. PT^{lower} cells projected sparsely to the lateral striatum. PT^{lower} neurons projected to the central amygdala and parasubthalamic nucleus, although these projections arise from cortical neurons outside of ALM (not shown). PT^{lower} neurons also made up the majority of the projection to the red nucleus, parabrachial nucleus, substantia nigra pars compacta, motor and sensory trigeminal nuclei in the hindbrain, and the pyramidal tract. Both cell types extended axon collaterals locally within the same sublamina as their somata, the subthalamic nucleus, zona incerta, and the midbrain reticular nucleus. PT^{upper} cells appeared to project more broadly to layer 1 in motor cortex. Mouse #1 and #2 were used for electrophysiological recordings and in both cases projections are labeled with Chr2.

Extended data figure 4



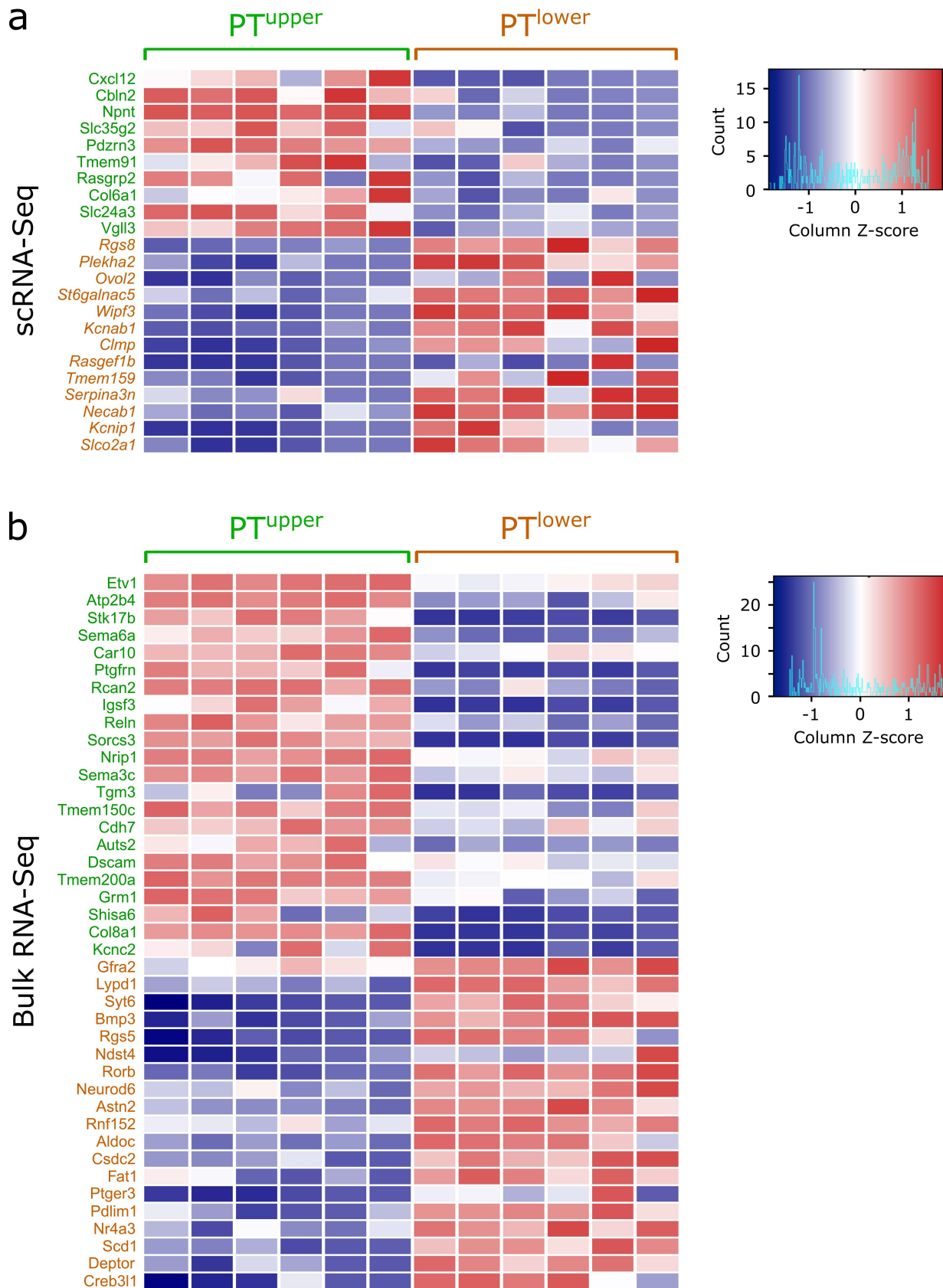
Extended data figure 4. Distribution of thalamus- and medulla-projecting PT neurons. **a.** The nuclei of PT neurons were labeled from the thalamus (green cells) and medulla (red cells) using AAVretro. Thalamus-projecting PT neurons are in upper L5b throughout motor cortex, whereas medulla-projecting PT neurons are in deep L5b. Schematics to the left of each image set are annotated coronal sections (Allen Reference Atlas) at approximately the same rostro-caudal level. **b.** AAVretro injection sites in the thalamus (*left*) and medulla (*right*).

Extended data figure 5



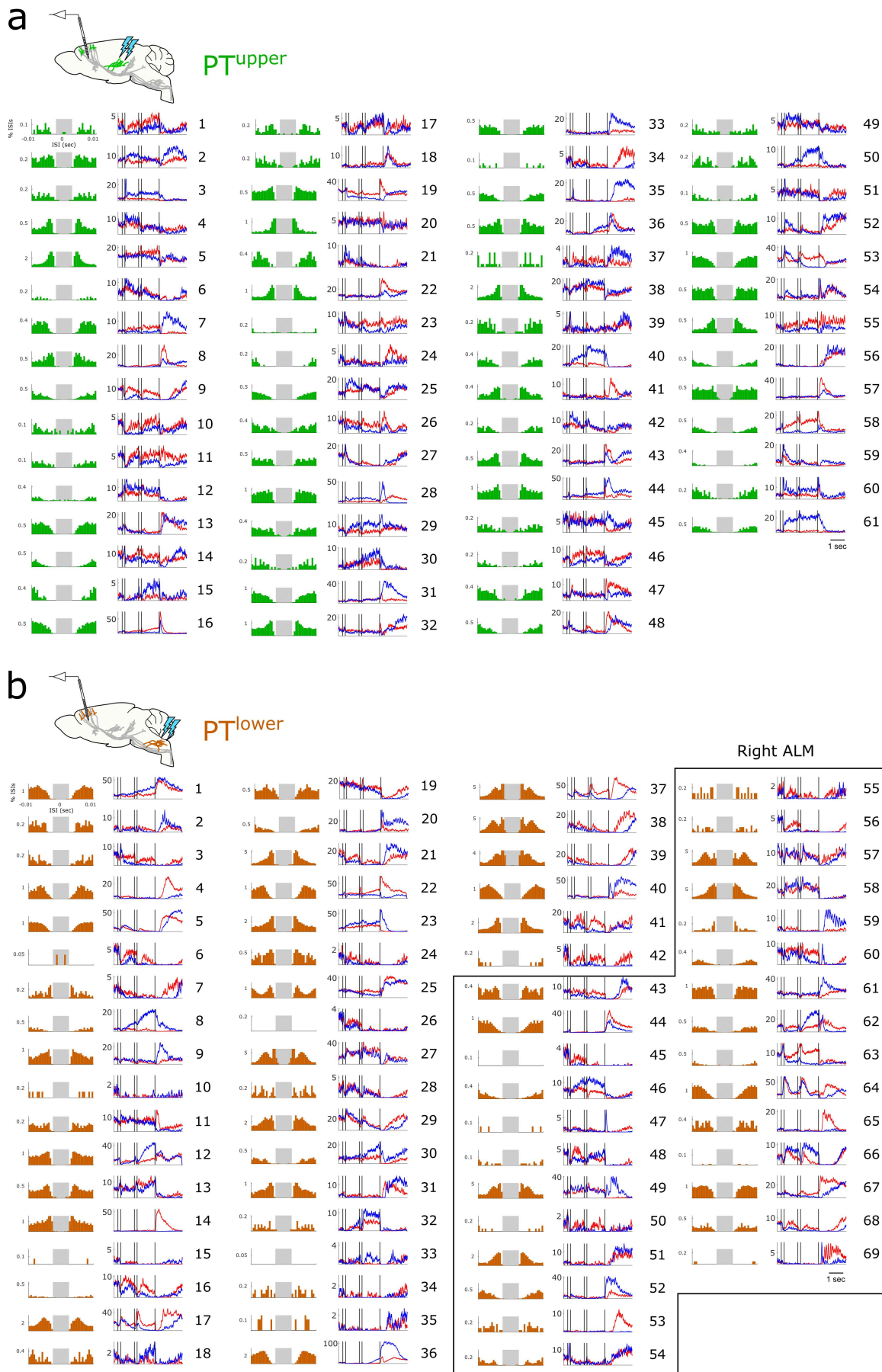
Extended data figure 5. Comparison of single cell RNA-Seq and bulk RNA-Seq. **a.** Scatter plot of PT^{upper} gene expression (measured as FPKM + 1, shown in log-scale) from scRNA-Seq (x-axis) and bulk RNA-Seq (y-axis) ($r = 0.86$). **b.** Same as (a) for PT^{lower} . **c.** Number of genes detected (**Methods**), from PT^{upper} neurons in bulk RNA-Seq and the union of scRNA-Seq measurements. The majority of genes were detected by both methods. **d.** Same as (c) for PT^{lower} . **e.** Total genes detected by bulk RNA-Seq and scRNA-Seq in PT^{upper} and PT^{lower} neurons. **f.** Number of genes detected in scRNA-Seq for each PT^{upper} and PT^{lower} neuron ('X', median; PT^{upper} , 9936 genes; PT^{lower} , 9865 genes).

Extended data figure 6



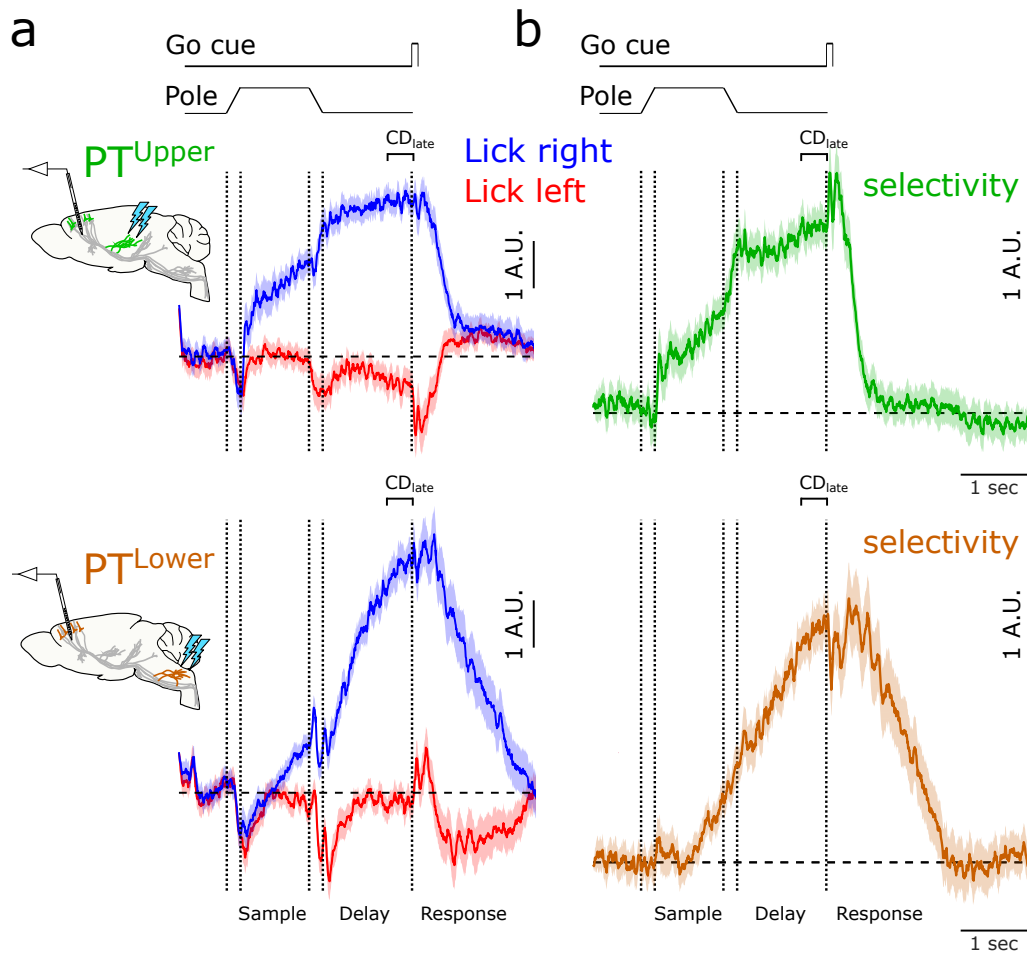
Extended data figure 6. Differentially expressed (DE) genes in PT^{upper} and PT^{lower} cells, based on bulk RNA-seq. a. Genes identified as DE in scRNA-Seq, assessed in the bulk RNA-Seq data. Rows represent genes, colored by differential scRNA-Seq expression in PT^{upper} (green) or PT^{lower} (brown). Columns in the heat map represent transcript expression in individual replicates, 6 for each of PT^{upper} and PT^{lower}. Colors show transcript intensity, as reflected from reads per kilobase of transcript per million mapped reads (RPKM), shown as Z-score. Pure blue shows replicates with very low expression (Z-score = -2); pure red shows replicates with very high expression (Z-score = +2). Bottom, distribution of z-scores. **b.** Same plot, for genes identified as DE from bulk RNA-Seq.

Extended data figure 7



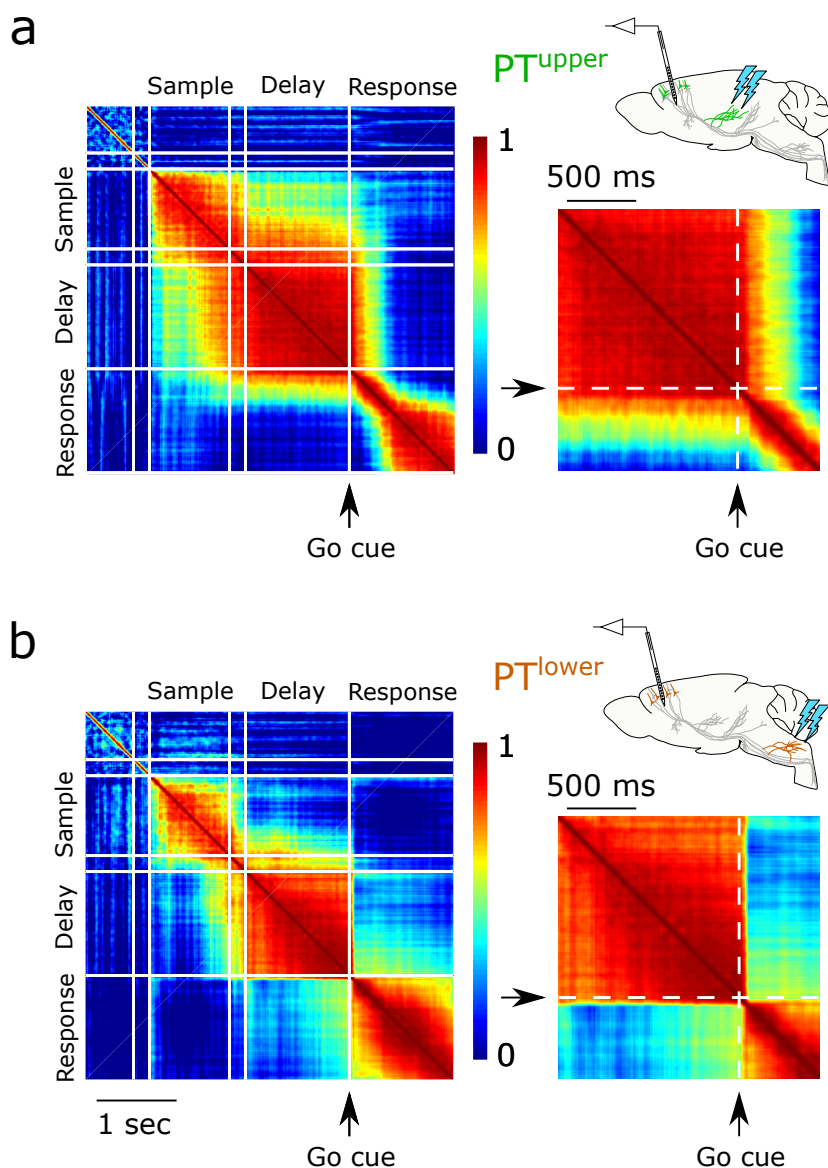
Extended data figure 7. Electrophysiology and trial-averaged spike rates for all identified PT neurons. **a.** PT^{upper} neurons. Left, inter-spike interval (ISI) histograms; right, trial-averaged activity on lick right (*blue*) and lick left trials (*red*). Gray shaded area in ISI histograms represents the interval of -2.5 ms to 2.5 ms. **b.** PT^{lower} neurons. Boxed region indicates neurons recorded in the right ALM (ipsilateral to injection site in medulla). All other neurons were recorded in the left ALM (contralateral to injection site).

Extended data figure 9



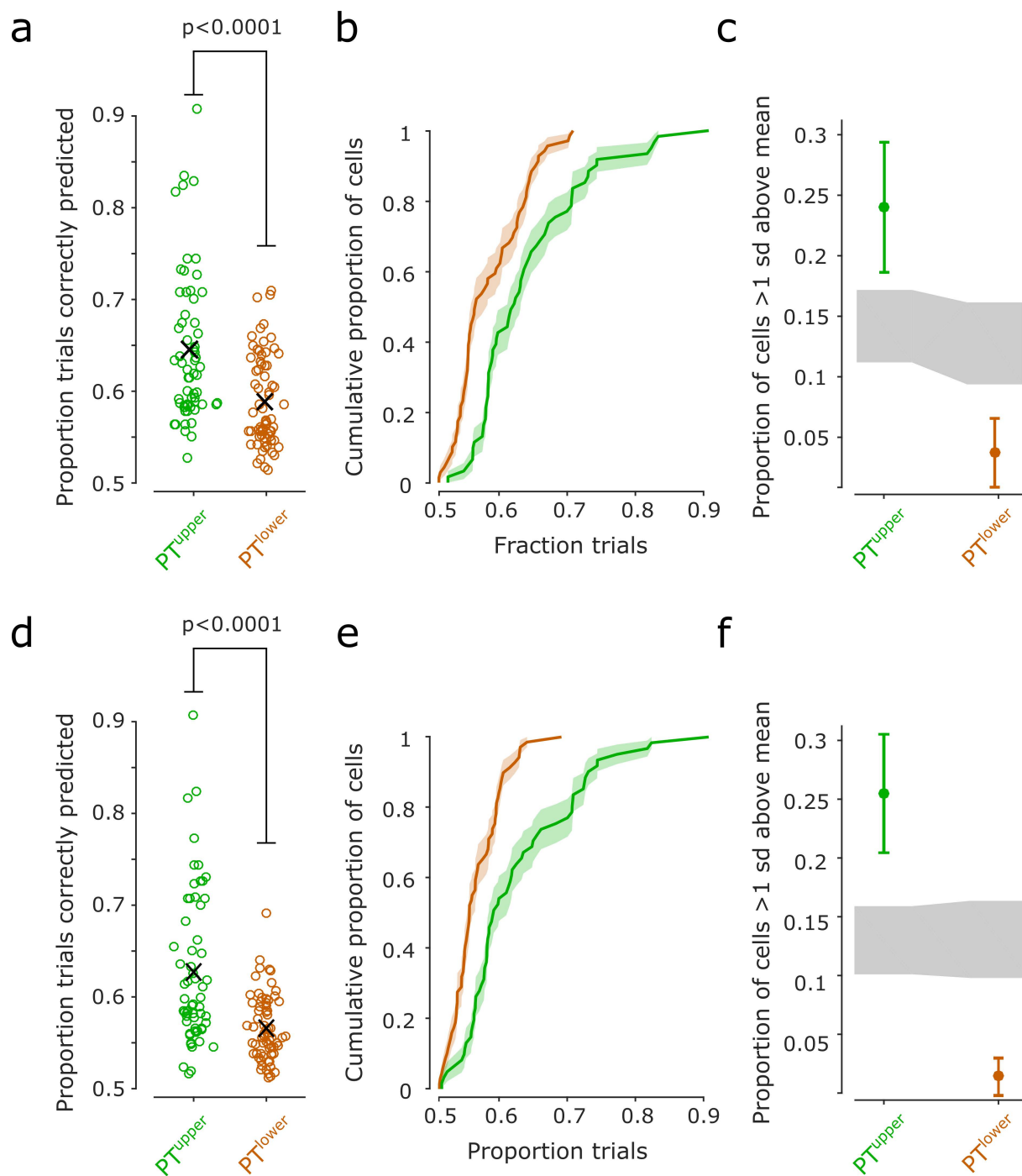
Extended data figure 9. Preparatory activity in the late delay epoch. **a.** Time course of the linear combination of neuronal activity that best differentiates trial types in the 400 ms immediately prior to the go cue (late coding dimension; CD_{late}) on lick right (*blue*) and lick left (*red*) trials for PT^{upper} (*top*) and PT^{lower} (*bottom*) neurons. **b.** Difference in CD_{late} projections on lick right and lick left trials (selectivity) in each population. Selectivity along CD_{late} is present in both populations, and persists after the go cue, but does not strongly modulate during movement initiation.

Extended data figure 10



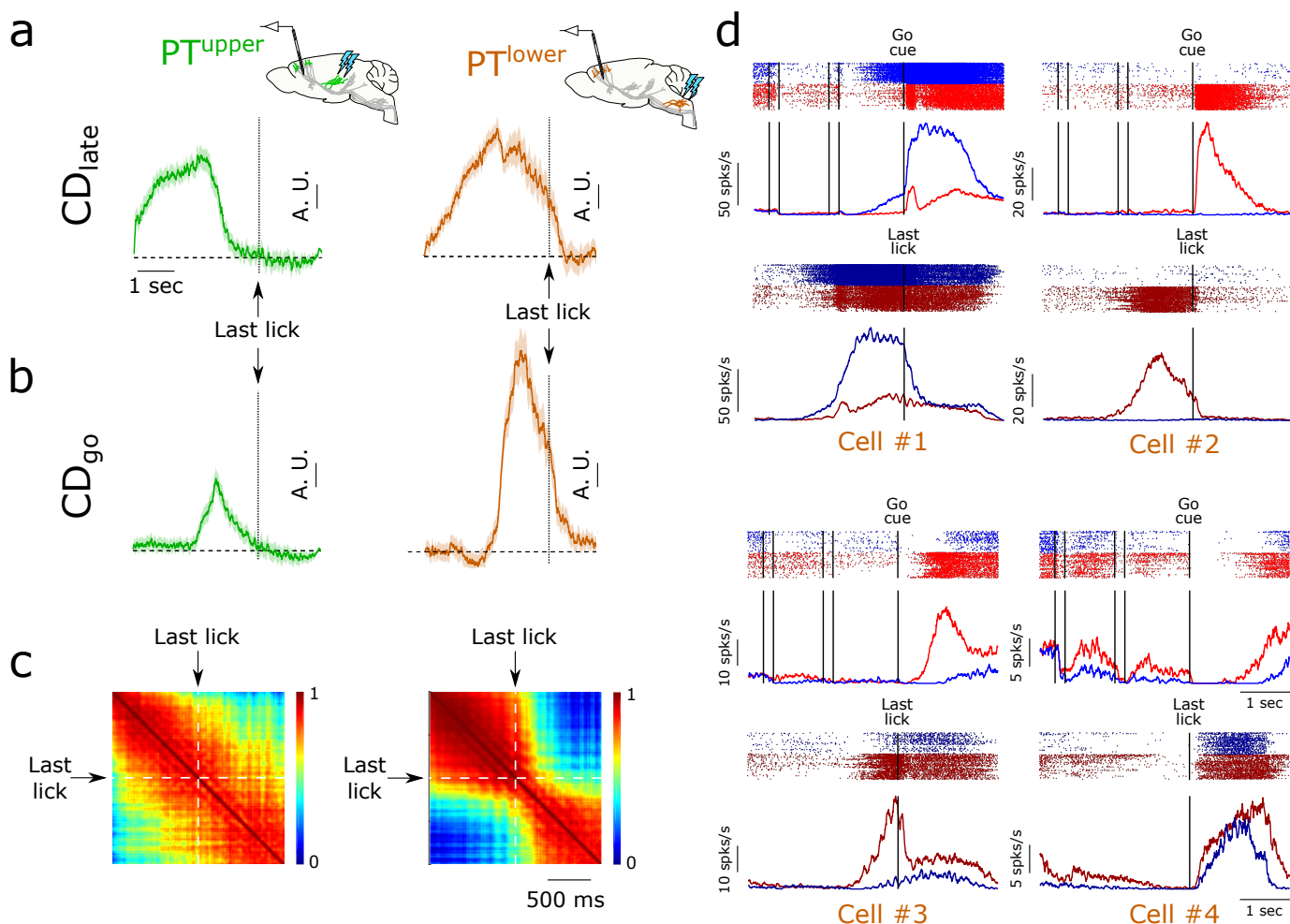
Extended data figure 10. Stability of the coding dimension (CD) across time within a trial. The coding dimension is the dimension that best discriminates trial types in a given time interval. Heat maps represent the correlation of the CD across all pairs of time points. **a.** In PT^{upper} neurons the coding dimension remains similar to CD_{early} for all time points preceding the go cue (including the delay epoch). **b.** In PT^{lower} neurons, the coding dimension in the delay epoch is largely orthogonal to the coding dimension in the sample epoch. Panels (a) and (b) show that the upcoming movement direction is encoded in a persistent manner in the PT^{upper} population, but not the PT^{lower} population. *Right panels:* Expanded view of the change in coding dimension around the time of the go cue. An abrupt change in the coding dimension occurs immediately after the go cue onset in the PT^{lower} population. A change also occurs in the PT^{upper} population, but more slowly (several hundred milliseconds), largely after initiation of movement.

Extended data figure 11



Extended data figure 11. Decoding of trial type in PT neuron types. **a.** Accuracy of trial type classification by single neurons in the 400 ms immediately following stimulus onset. 24.6% (15/61) of PT^{upper} neurons predicted trial type with at least 70% accuracy, whereas only 4.4% (3/69) of PT^{lower} neurons did so. Mean accuracy was also significantly higher in PT^{upper} neurons (PT^{upper}: $64.4 \pm 1.0\%$; PT^{lower}: $58.9 \pm 0.6\%$, mean \pm s.e.; $p < 0.0001$, two-sided Mann-Whitney U-test). **b.** Cumulative distribution function of the data in (a). **c.** Neurons containing the most trial-type information after stimulus onset disproportionately belong to the PT^{upper} class. The 10 most discriminative neurons all belonged to the PT^{upper} population. **d-f.** Same as (a-c) but decoding only based on spike rate increases above baseline. Trial-type selectivity during the sample epoch in PT^{lower} neurons was predominantly characterized by a modest suppression of spiking on one trial type, likely reflecting widespread lateral inhibition. Disregarding spike rate changes below baseline, no PT^{lower} neurons predicted trial type with at least 70% accuracy, while the same 24.6% of PT^{upper} neurons continued to do so and accounted for 20/21 of the most predictive neurons (PT^{upper}: $62.7 \pm 1.1\%$; PT^{lower}: $56.7 \pm 0.4\%$, mean \pm s.e.; $p < 0.0001$, two-sided Mann-Whitney U-test). As soon as the trial type is cued by the stimulus, upcoming movement direction is encoded robustly in the PT^{upper} population and only minimally in PT^{lower} cells.

Extended data figure 12



Extended data figure 12. Movement termination signals in PT^{lower} neurons. **a.** Selectivity along CD_{late} (same as **EDFig. 9**) for PT^{upper} (green; *left*) and PT^{lower} neurons (orange; *right*) aligned to the last lick in the response epoch. **b.** Selectivity along CD_{go} (same as **Fig. 6**) aligned to the last lick for each PT type. **c.** Correlation of coding dimension weights at all pairs of time points after the go cue for PT^{upper} neurons (*left*) and PT^{lower} neurons (*right*) using last-lick aligned spike rates. An additional transition in the population dynamics accompanies the termination of movement in PT^{lower} neurons, while there is no correlate of movement termination in PT^{upper} neurons. The change in dynamics at the offset of movement was somewhat less abrupt than at movement onset, likely a result of aligning data to the last lick port contact, which does not precisely mark the cessation of movement. **d.** Spike raster plots (*top*) and trial-averaged activity (*bottom*) for four example PT^{lower} neurons aligned to the go cue (lick right: *blue*; lick left: *red*) and the last lick port contact (lick right: *dark blue*; lick left: *dark red*).