1 Enrichment of specific GABAergic neuronal types in the mouse perirhinal cortex

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15 Abstract

16 GABAergic neurons represent 10-15% of the neuronal population of the cortex but exert a powerful
17 control over information flow in cortical circuits. GABAergic neurons show an extraordinary diversity in
18 their morphology, physiology, molecular markers and connectivity. This diversity allows GABAergic
19 neurons to participate in a wide variety of microcircuit motifs. The diversity of GABAergic neurons has
20 been shown to be conserved across cortical regions. The GABAergic population can be broadly divided
21 in three major classes parvalbumin, somatostatin and 5HT3aR groups. The largest GABAergic class in
22 the cortex is represented by the parvalbumin-expressing fast-spiking neurons, which provide powerful
23 somatic inhibition to their postsynaptic targets. Recently, the density of parvalbumin-expressing neurons
24 has been shown to be lower in associative areas of the mouse cortex, including the perirhinal cortex, as
25 compared to sensory and motor areas. In the present study we investigated whether this reduction in
26 parvalbumin-expressing neurons leads to a decreased GABAergic population, or to an enrichment of
27 other GABAergic cell-types. We found that the GABAergic population of the perirhinal cortex is
28 comparable to that of a primary sensory area, and it is enriched of neurons belonging to the 5HT3aR
29 group. We also demonstrate that, despite the low density of parvalbumin-expressing neurons, the
30 perirhinal cortex contains a comparable population of fast-spiking neurons, most of which do not express
31 parvalbumin. Our results demonstrate a yet uncharacterized diversity within the fast-spiking population
32 across cortical regions.

33

34 Introduction

35 The wide neuronal diversity of the cerebral
36 cortex is thought to endow it with a variety of
37 circuit motifs that shape information processing
38 (Harris and Shepherd 2015; Luo 2021). The
39 cerebral cortex can be parcellated in functional
40 areas that show specific cytoarchitectonic
41 features and molecular marker expression (van
42 Essen and Glasser, 2018). The relationship
43 between cortical parcellation and distribution of
44 neuron types in the cortex is poorly understood.
45 GABAergic neurons represent 10-15% of the
46 neuronal population and their diversity allows
47 them to participate in different cortical
48 microcircuits (Fishell and Kepecs, 2014;
49 Tremblay et al., 2021). GABAergic neurons are
50 divided in two major groups according to
51 embryonal origin: those derived from the medial
52 ganglionic eminence (MGE) and those derived
53 from the caudal ganglionic eminence (CGE)
54 (Rudy et al 2011). MGE-derived GABAergic

55 neurons are further divided according to
56 molecular marker expression into parvalbumin
57 (PV)-expressing and somatostatin (SST)-
58 expressing interneurons (INs). CGE-derived
59 interneurons (CGE-INs) are labeled by GFP in
60 the 5HT3aR-GFP mouse line (Lee et al 2010).
61 Transcriptomic analysis has confirmed this
62 classification and further divided the CGE group
63 into three major classes: vasoactive intestinal
64 peptide (VIP)-expressing, Lamp5, and Sncg
65 groups (Tasic et al 2018). Molecularly defined
66 GABAergic neurons have been shown to be
67 homogeneously distributed across sensory-
68 motor areas of the neocortex (Xu and Callaway
69 2010). However, a recent cortex-wide
70 examination of the distribution of three major
71 GABAergic groups (PV, SST, VIP) revealed
72 regional specializations (Whissell et al 2015;
73 Kim et al 2017). In particular, the density of PV-
74 INs decreases in association areas, including
75 the perirhinal cortex (PER). PV-INs are the most

76 abundant GABAergic type in the neocortex and
77 provide powerful perisomatic inhibition to their

78 postsynaptic targets (Tremblay et al., 2016).
79 PER is part of the parahippocampal region and

Figure 1. A. Representative confocal stack of immunofluorescence for GFP (turquoise) and PV (orange) in the wS1 of a GAD67-GFP mouse. **B.** Representative confocal stack of immunofluorescence for GFP and PV in PER of a GAD67-GFP mouse. **C.** Representative confocal stack of immunofluorescence for GFP (turquoise) and SST (orange) in the wS1 of a GAD67-GFP mouse. **D.** Representative confocal stack of immunofluorescence for GFP and SST in PER of a GAD67-GFP mouse. **E.** Graph plotting the samples in the principal component space using PC1 and PC2. **F.** Bar plot showing the statistical comparison of the density of GABAergic neurons across the layers of A35 (red), A36 (yellow) and wS1 (purple) (n= 5 mice): L1 (F= 1.33, df=2, p= 0.29, One-Way ANOVA); L2/3 (F= 37.4, df= 2, p= 6.9E-6, One-Way ANOVA; A35 vs wS1, p= 7.6E-6, Bonferroni correction; A36 vs wS1, p= 0.0002, Bonferroni correction); L5 (F= 2.8, df= 2, p= 0.1, One-way ANOVA); L6 (F= 11.5, df= 2, p= 0.002, One-Way ANOVA; A35 vs wS1, p= 0.008, Bonferroni correction). **G.** Cumulative percentage of SST-IR (red), PV-IR (black) and GFP-IR/PV-SST neurons in the total GABAergic population of A35. **H.** Cumulative percentage of SST-IR (red), PV-IR (black) and GFP-IR/PV-SST- neurons in the total GABAergic population of A36. **I.** Cumulative percentage of SST-IR (red), PV-IR (black) and GFP-IR/PV-SST- neurons in the total GABAergic population of wS1.

80 represents a gateway for sensory information
81 entering the hippocampal formation (Witter et
82 al., 2000), by exerting an inhibitory control on
83 the information travelling into the hippocampal
84 network (DeCurtis and Paré, 2004). The
85 mechanisms underlying this inhibitory control
86 are still poorly understood. One possibility is that
87 this inhibitory gating relies on strong perisomatic
88 feedforward inhibition mediated by PV-INs
89 (Willems et al 2018). This is in striking contrast
90 with the low density of PV-INs in PER. The low
91 density of PV-INs in PER suggests that either
92 the GABAergic population of PER is smaller
93 than that of the neocortex, or that other
94 GABAergic cell-types are enriched. In the
95 present study we aimed to explore these two
96 scenarios by combining mouse genetics,
97 molecular marker expression, enhancer
98 mediated transgene expression and
99 electrophysiological characterization of
100 GABAergic neurons in PER. We compared the
101 GABAergic population of PER to that of the area
102 of whisker representation of the primary
103 somatosensory cortex (wS1), whose
104 GABAergic diversity has been extensively
105 described (Lee et al., 2010; Feldmeyer et al.,
106 2018). We found that indeed the GABAergic
107 population is not smaller in PER and contains a
108 higher density of 5HT3aR-expressing
109 interneurons. Characterization of the firing
110 patterns of GABAergic neurons in PER shows
111 that this is partially due to an enrichment of late-
112 spiking neurogliaform cells. Most strikingly, we
113 also found that the percentage of fast-spiking
114 neurons was much higher than expected from
115 the density of PV-INs. Using an enhancer-driven
116 viral approach, we demonstrate that the
117 population of fast-spiking neurons in PER

118 includes PV-expressing neurons and a
119 population of neurons not expressing PV.

120

121 Results

122

123 Comparison of the GABAergic population of 124 wS1 and PER

125 To compare the whole GABAergic population of
126 PER and wS1 we used the GAD67-EGFP
127 mouse line and performed immunofluorescence
128 staining for PV, SST and GFP (Figure 1A-D).
129 We quantified the number of PV-IR, SST-IR and
130 GFP-IR/PV-SST- (neurons labeled by the
131 GAD67-EGFP mouse line but not expressing
132 either PV or SST) neurons across layers of the
133 two cortical areas and calculated their densities.
134 We performed a PCA on the densities of these
135 markers across the layers of wS1 and PER (A35
136 and A36) to test how these cell-types describe
137 these two cortical regions (Figure 1E). The first
138 two principal components accounted for 93.8%
139 of the variance and the data was well
140 segregated in the principal component space
141 (Figure 1E and Figure 1 supplement 2A). The
142 variables that best explained the variance were
143 the density of PV-INs in layers 2/3-6, which was
144 lower in PER, and the density of GFP-IR/PV-
145 SST- in layers 2/3-6, which was higher in PER
146 (Figure 1E and Figure 1 – figure supplement
147 2B). The PCA results were confirmed by a
148 statistical comparison of the three molecularly
149 defined GABAergic populations (Figure 1 –
150 Figure supplement 3). Indeed, the density of PV-
151 INs was significantly lower in layer 2/3-6 of PER,
152 whereas the density of GFP/PV-SST neurons
153 was significantly higher in L2/3-6 as compared
154 to wS1 (Figure 1 – Figure supplement 3). The
155 PCA analysis showed a slight contribution of
156 SST-INs in L2/3, and indeed their density was

Figure 2. A. Representative confocal stack of immunofluorescence for GFP (green) in wS1 of the 5HT3aR-EGFP mouse line. B. Representative confocal stack of immunofluorescence for GFP (green) in PER of the 5HT3aR-EGFP mouse line. C. Bar plot showing the statistical comparison of the density of GFP-IR neurons in the 5HT3aR-EGFP mouse line (n= 10 slices in each area): L1 (F= 0.78, df= 2, p= 0.47, One-way ANOVA); L2/3 (F= 10.81, df= 2, p= 0.0004, One-way ANOVA; A35 vs wS1, p= 0.0003, Bonferroni correction; A36 vs wS1, p= 0.009, Bonferroni correction); L5 (F= 13.24, df= 2, p= 9.84E-5, One-way ANOVA; A35 vs wS1, p= 0.0005, Bonferroni correction; A36 vs wS1, p= 0.001, Bonferroni correction); L6 (F= 10.02, df= 2, p= 0.0006, One-way ANOVA; A35 vs wS1, p= 0.002, Bonferroni correction; A36 vs wS1, p= 0.0009, Bonferroni correction).

157 significantly higher in L2/3 and 6 of PER (Figure 158 1E and Figure 1 – Figure supplement 3). Our 159 data show that A35 and A36 are very similar in 160 their GABAergic population. However, the 161 densities of these GABAergic types in A36 are 162 between that of A35 and wS1 (Figure 1E and F, 163 Figure 1 – Figure supplement 3). These results 164 confirmed previous reports of low density of PV- 165 INs in PER (Whissel et al., 2015; Kim et al., 166 2017), and further show that PER is enriched in 167 a population of GABAergic neurons that does 168 not express either PV or SST.

169 Finally, we show that PER has a higher density 170 of GABAergic neurons, particularly in L2/3 and 171 L6 (Figure 1F). Our results suggest that in PER 172 the low density of PV-INs is compensated by an 173 enrichment of other classes of GABAergic 174 neurons. A comparison of the percentage of 175 marker-IR neurons in the GABAergic population 176 further confirmed the reduction of PV-INs and an 177 increased representation of GFP-IR/PV-SST 178 neurons across the layers of A35 and A36 179 (Figure 1G-I).

180 181 PER is enriched in GABAergic cell-types of 182 the 5HT3aR group

183 In sensory and motor areas of the dorsal cortex, 184 PV-INs represent the largest group of 185 GABAergic neurons (Tremblay et al., 2016). Our 186 results show that in PER the largest population

187 across layers does not express either PV or SST 188 (Figure 1). We reasoned that this population 189 might be in part composed of neurons 190 expressing GFP in the 5HT3aR-EGFP mouse 191 line, which do not express either PV or SST (Lee 192 et al., 2010). We tested this hypothesis by 193 quantifying the density of GFP labeled neurons 194 in the 5HT3aR-EGFP mouse line (Gong et al., 195 2003) (Figure 2). We found that PER contains a 196 significantly higher density of GFP labeled 197 neurons as compared to wS1, particularly in 198 L2/3, 5 and 6 (Figure 2C). We then tested 199 whether VIP-INs contributed to this difference 200 by performing immunolabelling for VIP in the 201 GAD67-EGFP mouse line (Figure 2 – Figure 202 supplement 1). The density of VIP-INs in PER 203 tended to be higher than in wS1, but the 204 difference was not significant, other than in L5 205 (Figure 2 – Figure supplement 1). We conclude 206 that the VIP-INs are not the major source of the 207 enrichment of 5HT3AR-INs in PER.

208 209 L5 of PER is enriched of late-spiking 210 neurogliaform neurons

211 The 5HT3AR-INs group includes VIP-INs, 212 Lamp5-INs (neurogliaform), and sncg-INs 213 (CCK-basket cells) (Tasic et al., 2018). Our 214 results show that VIP-INs provide only a small 215 contribution to the enrichment in 5HT3AR-INs in 216 PER (Figure 2 – Figure supplement 1). The

Figure 3. A. Firing patterns recorded from GABAergic neurons in L5 of A35 (upper row) and wS1 (lower row). **B.** Confocal stacks showing of a neurogliaform cell in PER (left) and wS1 (right). **C.** Comparison of the percentage of firing patterns (left) and molecularly defined populations (right) in L5 of A35. Note the high percentage of fast-spiking neurons as compared to the low percentage of PV-INs in PER.

217 other two groups lack specific mouse lines or
 218 unique molecular markers. We used instead in
 219 vitro electrophysiology to characterize the firing
 220 patterns of GABAergic interneurons in L5 of
 221 PER. We focused on L5 because it is where we
 222 found the largest difference in the percentage of
 223 GABAergic neurons not expressing PV and SST
 224 between PER and wS1 (Figure 1G-I).
 225 Neurogliaform cells in the cortex, hippocampus
 226 and amygdala show a “late-spiking” phenotype
 227 characterized by a long latency to the first spike,
 228 broad AHPs and no adaptation (Overstreet-
 229 Wadiche and McBain, 2015). Sncg-INs include
 230 CCK-basket cells, and show a regular firing
 231 pattern with little or no adaptation (Kawaguchi
 232 and Kubota, 1998). We obtained brain slices
 233 containing PER from GAD67-GFP mice and
 234 performed whole-cell patch clamp experiments
 235 in current clamp to measure voltage responses
 236 of GABAergic neurons. We found four types of
 237 firing patterns in PER (n= 33 cells): the fast-

238 spiking phenotype (51.5%), associated to PV-
 239 INs, the irregular firing pattern (3.03%),
 240 associated to some VIP-INs, the late-spiking
 241 phenotype (39.4%), associated to neurogliaform
 242 cells, and the adapting firing pattern (6.07%),
 243 associated to some SST-INs. In L5 of wS1 we
 244 found five firing patterns (n= 20 cells): fast-
 245 spiking (55%), irregular (5%), late-spiking
 246 (10%), adapting (5%), quasi-fast spiking (25%),
 247 associated to layer 4 targeting non-Martinotti
 248 SST-INs (Ma et al., 2006; Nigro et al., 2018)
 249 (Figure 3A). Our electrophysiological
 250 characterization of the GABAergic population in
 251 PER and wS1 showed that late-spiking
 252 neurogliaform cells are enriched in L5 of PER.
 253 The late-spiking neurons in PER had short
 254 dendrites and a dense local axonal arborization,
 255 typical of neurogliaform cells in neocortical L1
 256 and hippocampus (Overstreet-Wadiche and
 257 McBain, 2015) (Figure 3B). We conclude that
 258 neurogliaform cells might account for most of

259 the difference in the density of 5HT3aR-INs
260 between PER and wS1.

261

262 **A fast-spiking population that do not**
263 **express PV in PER**

264 Unexpectedly, the fast-spiking phenotype
265 accounted for the largest population in PER and
266 wS1, which is at odds with the low density of PV-
267 INs in PER (Figure 1 and Figure 3C). We
268 hypothesized that some fast-spiking neurons in
269 PER do not express detectable levels of PV. To
270 test this hypothesis, we labelled fast-spiking
271 neurons with a viral approach that exploits
272 enhancer driven expression of tdTomato in PV-
273 INs throughout the brain (Vormstein-Schneider

274 et al., 2020). We injected the S5E2-dTom virus
275 in PER of GAD67-EGFP mice and stained for
276 SST to subtract the population of virus
277 transfected neurons that co-expressed SST
278 (Figure 4 – Figure supplement 1 and Figure 4 –
279 Figure supplement 2). We found that the E2-
280 dTom/SST- represented the largest GABAergic
281 population in the deep layers of PER (Figure 4A-
282 B). The percentage of E2-dTom/SST- was 47.1
283 $\pm$ 11.8% of the total GABAergic population in L5
284 of A35, which was very similar to the percentage
285 of PV-INs in L5 wS1 (48.3 $\pm$ 8.3%) and to the
286 percentage of fast-spiking neurons in L5 of PER
287 (51.5%) (Figure 4C).

Figure 5. A. Confocal stack showing the location in L5 of A35 of a E2-dTom neuron filled with biocytin (green). Neurons labeled by the E2-dTom virus are shown in red. Dashed line shows the border with LEC. **B.** Pie chart showing the percent of LTS (orange), adapting (red) and fast-spiking (FS) (black) among E2-dTom neurons. **C-E.** Confocal stacks of a E2-dTom neuron (D, red) filled with biocytin (C, green) and expressing PV (E, white, white arrow). **F.** Voltage response of the cell shown in C-D to hyper- and depolarizing current steps. **G-I.** Confocal stacks of a E2-dTom neuron (H, red) filled with biocytin (G, green) and not expressing PV (I, white, white arrow). **J.** Voltage response of the cell shown in C-D to hyper- and depolarizing current steps.

288 This result further suggests that PER is
289 populated by a population of GABAergic
290 neurons expressing a fast-spiking phenotype
291 but not expressing PV. To directly test this
292 hypothesis, we performed in vitro patch clamp
293 recordings from brain slices of wild type C57
294 mice injected with the S5E2-dTom virus. We
295 found that 88.9% (16 out of 18 neurons) of
296 dTomato labelled neurons showed a fast-

297 spiking phenotype and 11.2% showed an
298 adapting (1/18) or low-threshold firing pattern
299 (1/18), typical of SST neurons (Figure 5A-B, and
300 Figure 5 – Figure supplement 1). We performed
301 immunofluorescence for PV on 15 of the
302 recorded fast-spiking neurons and found that
303 40% (6/15) did not express PV (E2-PV-) (Figure
304 5C).

305 Our results show that the fast-spiking population
306 of PER contains neurons that do not express
307 PV, highlighting a novel diversity within this
308 GABAergic type that is area specific.

Figure 6. A. Dendrogram obtained from a hierarchical cluster analysis of 44 fast-spiking neurons (11 in wS1 and 33 in PER). The dashed line represents the level at which the clusters were identified by using the inconsistency index (see Methods). Numbers on the x-axis represent the cell's IDs color-coded according to the cortical area (yellow, PER; purple, wS1). Filled diamonds indicate E2-dTom neurons expressing PV. Open diamonds indicate E2-dTom neurons not expressing PV. **B.** Representative voltage response of fast-spiking neurons to a -100 pA current step in wS1 (purple) in PER (yellow) neurons. The dashed line indicates the peak of the response. The plot on the right shows the IR of all neurons (circles) and the population mean (black line) (wS1, 145.4 ± 46.4 M Ω ; PER, 310.7 ± 114.4 M Ω ; $p = 3.6E-8$, T-test). **C.** Representative voltage response to a hyperpolarizing current step of fast-spiking neurons in wS1 (purple) and in PER (yellow). All responses used to calculate the sag ratio reached -90 ± 2 mV. Note the difference in the steady-state response (dashed line). The plot on the right shows the sag ratio of all neurons (circles) and the population average (black line) (wS1, 0.78 ± 0.1 ; PER 0.94 ± 0.02 ; $p = 0.0001$, T-test). **D.** Representative action potentials of fast-spiking neurons in wS1 (purple) and PER (yellow). The plot on the right shows the AP-HW of all neurons (circles) and the population mean (black line) (wS1, 0.28 ± 0.03 ms; PER, 0.42 ± 0.07 ms; $p = 2.9E-12$, T-test). **E.** Representative AHPs following single APs in wS1 (purple) and PER (yellow). The AHPdur is indicated by the two dashed lines. The plot on the right shows the AHPdur of all neurons (circles) and the population mean (black line) (wS1, 29.1 ± 6.2 ms; PER, 51.8 ± 21.3 ms; $p = 2.4E-6$, T-test). **F.** Representative responses of fast-spiking neurons in wS1 (purple) and PER (yellow) to depolarizing current steps eliciting maximal firing frequency. The plot on the right shows the Fmax in all neurons (circles) and the population mean (black line) (wS1, 194.8 ± 60.7 APs/s; PER, 109.5 ± 29.6 APs/s; $p = 0.0008$, T-test). **G.** Plot of the average number of APs evoked with current steps of increasing amplitude in wS1 (purple) and PER (yellow).

309 310 **Fast-spiking interneurons in PER show** 311 **different electrophysiological properties** 312 **than in wS1**

313 To test whether the firing patterns of fast-spiking
314 neurons not-expressing PV in PER are different
315 from PV-INs in PER and wS1, we performed a
316 hierarchical cluster analysis on the
317 electrophysiological properties (Figure 6A). The
318 dendrogram shows that the population analyzed
319 can be divided in two clusters, one containing all
320 wS1 neurons, and a second containing all but 4
321 PER neurons (Figure 6A). The group containing
322 perirhinal fast-spiking neurons included PV-INs
323 and neurons not expressing PV, suggesting that
324 these neurons show similar electrophysiological
325 responses (Figure 6A). We further compared
326 the electrophysiological parameters included in
327 the cluster analysis (see Methods) and found
328 that fast-spiking interneurons in PER had a
329 significantly higher IR and as a consequence
330 they fired action potentials in response to
331 smaller current steps (Figure 6B and G). Fast-
332 spiking neurons in PER had a less pronounced
333 sag, which suggests a lower expression of HCN
334 channels (Figure 6C). A hallmark of PV-INs are
335 their fast APs and high firing rate, interestingly
336 fast-spiking neurons in PER had wider APs,
337 longer AHP and reached lower maximal firing
338 rates than their wS1 counterparts (Figure 6D-
339 G). We did not find any difference between E2-
340 PV+ and E2-PV- neurons in PER, suggesting
341 that these neurons might belong to the same
342 type and differ only in expression of PV.

343

344 **Discussion**

345 The present study offers a comprehensive
346 description of the GABAergic diversity in the
347 mouse PER, and demonstrates several
348 differences compared to a sensory region
349 (wS1). First, we demonstrate that PER contains
350 a comparable density of GABAergic neurons as
351 other cortical areas despite the low density of
352 PV-INs. Second, PER is enriched in
353 neurogliaform cells in layer 5. Since our
354 molecular characterization showed no other
355 candidate for the increased density of 5HT3aR-
356 INs in other layers, we hypothesize that
357 neurogliaform cells are more numerous
358 throughout the layers of PER. Third, we
359 corroborated previous observations of an
360 increased SST-INs/PV-INs ratio in L2/3 (Kim et
361 al., 2017). We extend this observation by
362 showing that the density of SST-INs is
363 significantly higher in PER as compared to wS1.
364 Finally, we demonstrate that the fast-spiking
365 population of PER is comparable to that of wS1
366 and most of these neurons do not express PV.
367 We then show that the fast-spiking population of
368 PER shows several electrophysiological
369 differences with that in wS1 (and other
370 telencephalic areas), highlighting a novel
371 diversity within this electrophysiological cell-
372 type.

373

374 **Enrichment of 5HT3aR-INs in PER**

375 We found that, although the density of PV-INs is
376 lower in PER than in wS1, the GABAergic
377 population is of comparable size, or even larger
378 (e.g., in L2/3 and 6). This difference is partially

379 due to an enrichment of GABAergic neurons
380 belonging to the neurogliaform type, and to a
381 lesser extent to the VIP-expressing group.
382 These GABAergic types belong to the CGE-
383 derived class (Tremblay et al., 2016).
384 Interestingly, transcriptomic analysis of the
385 human temporal cortex showed that the human
386 cortex is enriched of CGE-INs and
387 intratelencephalic projecting excitatory cells (IT)
388 (Hodge et al., 2019). CGE-INs and IT-projection
389 neurons show a preferential connectivity that is
390 established during development (Wester et al.,
391 2019). The PER shows very sparse projections
392 outside of the telencephalon, except for the
393 thalamus, which suggests that a developmental
394 program might be responsible for the
395 enrichment of CGE-INs (McIntyre et al., 1996;
396 Benavidez et al., 2021).

397 398 **Most fast-spiking GABAergic neurons in** 399 **PER do not express PV**

400 The low expression of PV in PER has been used
401 by neuroanatomists to delineate PER for
402 several decades in different species (Pitcäen
403 and Amaral, 1993; Burwell et al., 1995; Uva et
404 al., 2004; Beaudin et al., 2013). However, this
405 observation has only recently been examined in
406 the context of GABAergic cell-types and
407 inhibitory circuits using specific mouse lines
408 targeting PV-INs (Whissel et al., 2015; Kim et
409 al., 2017). We have recently described that the
410 labelling in the PV-IRES-Cre mouse line is very
411 inefficient and quantifications of the number of
412 PV-INs would be dramatically underestimated
413 (Nigro et al., 2021). The present study
414 corroborates the low density of PV-INs in PER
415 by quantifying PV-IR neurons in PER and wS1.
416 However, our electrophysiological
417 characterization demonstrates that fast-spiking
418 interneurons represent half of the GABAergic
419 population in PER, similarly to wS1. Taking
420 advantage of a newly developed viral approach
421 we further demonstrate that most fast-spiking
422 neurons in PER do not express PV. The
423 electrophysiological properties of fast-spiking
424 interneurons in PER differ from those in wS1.
425 Our results suggest that the fast-spiking
426 population of the cortex shows a yet
427 unappreciated diversity. Recent transcriptomic
428 examination of the GABAergic cell-types of the
429 whole cortex did not identify transcriptomic
430 types with signatures of fast-spiking neurons but
431 not expressing PV (Yao et al., 2021). This is

432 likely due to the use of specific mouse lines
433 targeting different GABAergic types, which
434 would prevent the identification of a population
435 that do not express any of the main known
436 GABAergic molecular markers.

437

438 **Ideas and hypotheses**

439 The expression of PV is the hallmark of fast-
440 spiking GABAergic neurons throughout the
441 telencephalon (Yao et al., 2021). Our results
442 raise the hypothesis that PV expression per se
443 might not be enough to define this GABAergic
444 class in all cortical areas. We have previously
445 shown that the PV-IRES-Cre mouse line shows
446 a low efficiency in several associative areas of
447 the cortex (Nigro et al., 2021). The low density
448 of PV-INs seems also to be a common feature
449 of associative areas (Whissel et al., 2015; Kim
450 et al., 2017). We hypothesize that our results
451 might extend beyond PER to other associative
452 areas of the cortex. Transcriptomic analysis of
453 neurons labeled by the S5E2-dTom virus might
454 reveal the taxonomic relationship between
455 these different types of fast-spiking neurons in
456 the cortex.

457 Until now we have assumed that fast-spiking
458 neurons not expressing PV belong to the PV
459 GABAergic class based on our
460 electrophysiological characterization. The lack
461 of PV expression might be due to plasticity of the
462 circuit in which these cells are embedded.
463 Indeed, PV expression is activity dependent and
464 has been shown to be modulated by learning
465 (Donato et al., 2013).

466 An alternative scenario could be that these
467 neurons belong to a different class (e.g., Lamp5,
468 scng, SST, or VIP), and simply lost their
469 characteristic marker. Interestingly, the S5E2-
470 dTom virus labels all PV-INs and about 60% of
471 the SST population. The fast-spiking phenotype
472 described here in perirhinal neurons is
473 reminiscent of the quasi-fast spiking phenotype
474 of non-Martinotti neurons described in L4 and
475 L5b of wS1 (Ma et al., 2006; Nigro et al. 2018;
476 Naka et al. 2019). However, our
477 characterization of the firing pattern of SST
478 neurons in L5 of PER shows that these neurons
479 express either an adapting or a low-threshold
480 spiking phenotype. Moreover, non-Martinotti
481 neurons are extremely rare outside of wS1 (Ma
482 et al., 2006; Scala et al., 2019). Finally, some
483 chandelier cells do not express PV, and express
484 a firing pattern similar to that described here in

485 PER (Taniguchi et al., 2013). For these reasons
486 we believe that the fast-spiking population in
487 PER belongs to the PV transcriptomic class but
488 lacks PV expression.

489

490 Methods

491 Animal models

492 The research described here was performed on
493 adult mice (>8 weeks old) of both sexes. We
494 used the following mouse lines: C57BL/6J line
495 from Jackson Laboratories (stock 000664),
496 GAD67-eGFP obtained as gift from Dr.
497 Yanagawa (Tamamaki et al., 2003), the PV-
498 IRES-Cre line (B6;129P2-Pvalbtm1(cre)arbr/J,
499 stock 008069); R26R-EYFP (129X1-
500 Gt(ROSA)26Sortm1(EYFP)Cos/J stock
501 006148) and the SST-Cre line
502 (Ssttm2.1(cre)Zjh/J, stock 013044). We used
503 two brains of the 5HT3aR-EGFP mouse line
504 (MMRRC: Tg(Htr3a-EGFP)DH30Gsai/Mmnc
505 stock: 000273-UNC) kindly donated by Dr.
506 Ramesh Chittajallu. The PV-IRES-Cre line was
507 crossed with the R26R-EYFP to obtain EYFP
508 expression in PV-INS. The PV-IRES-Cre was
509 bred as homozygous, whereas the GAD67-
510 eGFP and the R26R-EYFP were bred as
511 heterozygous. Animals were bred in house,
512 group housed in enriched cages with water and
513 food ad libitum. Animals were kept with an
514 inverted dark/light cycle of 12h/12h.

515

516 AAV2/1-S5E2-dTomato (S5E2-dTom) 517 purification

518 The plasmid construct for the S5E2-dTom was
519 purchased from Addgene (#135630)
520 (Vormstein-Schneider et al., 2020). We
521 packaged the construct in AAV 2/1 serotype (a
522 mosaic of capsid 1 and 2) and generated
523 injection-grade viral vector using Heparin
524 column affinity purification method (Nair et al
525 iScience 2020). All the plasmids for AAV vector
526 preparations were made using endotoxin free
527 plasmid maxiprep kit (#12663, Qiagen). AAV
528 293 cells (#CVCL_6871, Agilent, USA) used for
529 vector productions were maintained in DMEM (#
530 41965062, Thermo Fisher Scientific) containing
531 10% fetal bovine serum (#16000-044,
532 ThermoFisher, USA) and penicillin/streptomycin
533 antibiotics (#15140122, Thermo Fisher
534 Scientific). The day before transfection, we
535 seeded approximately 7×10^6 AAV 293 cells on
536 150 mm cell culture plates and maintained at
537 37°C, 5% CO₂. For the calcium chloride

538 mediated co-transfection we used 22.5 µg
539 pAAV-containing the transgene, 22.5 µg
540 pHelper (#240071, Agilent, USA), 11.3 µg pRC
541 (#240071, Agilent, USA) and 11.3 µg pXR1
542 (NGVB, IU, USA) capsid plasmids. The culture
543 medium was replaced after 7 hours with fresh
544 10% FBS containing DMEM. Transfected cells
545 were scrapped out after 72 hours and
546 centrifuged at 200xg. The pellet was subjected
547 to lysis using a buffer containing 150 mM NaCl-
548 20 mM Tris pH 8.0 and 10% sodium deoxy
549 cholate. The lysate was then treated with
550 Benzonase nuclease HC (#71206-3, Millipore)
551 for 45 minutes at 37 °C. Subsequently, the
552 benzonase treated lysate was centrifuged at
553 3000xg for 15 mins. The clear supernatant was
554 then subjected to HiTrap® Heparin High
555 Performance (#17-0406-01, GE) affinity column
556 chromatography using a peristaltic pump. The
557 elute from the Heparin column was then
558 concentrated using Amicon Ultra centrifugal
559 filters (#Z648043, Millipore). We performed
560 quantitative PCR on the viral stock and the titer
561 was determined as approximately 10¹¹
562 infectious particles/ml.

563

564 Injections of S5E2-dTomato viral vector

565 The AAVs were injected into the brains of C57
566 (n= 6), GAD67-EGFP (n= 2) and on PV-EYFP
567 (n= 2) mice. Mice were anaesthetized with
568 isoflurane (4% Nycomed, airflow 1 l/min) in an
569 induction chamber. The mice were placed in a
570 stereotactic frame on a heated pad (37 °C)
571 throughout the procedure and head-fixed with
572 ear bars. Eye ointment was applied to protect
573 the cornea. The following analgesic were
574 applied subcutaneously: buprenorphine
575 hydrochloride (0.1 mg/Kg, Temgesic, Invidior),
576 meloxicam 1 mg/Kg, Metacam Boehringer
577 Ingelheim Vetmedica), bupivacaine
578 hydrochloride (1 mg/Kg locally, Marcain, Astra
579 Zeneca). The skin on the incision site was
580 shaved and disinfected with Pyrissept. An
581 incision was made to access the skull. The skull
582 was thinned with a dental drill at the desired
583 location, and a hole was punched with the tip of
584 a glass pipette. We used the following
585 coordinates from bregma: for wS1 -0.7 mm AP,
586 +3.1 mm ML, -0.15 and 0.55 DV; for PER -3 mm
587 AP, -1.5 DV, for the ML the pipette was moved
588 to the edge of the skull and then retracted 0.4
589 mm. Injections of 100-200 nl (50 nl/minute) in
590 each location were made with a glass pipette

591 (20-30 μm tip size) attached to an injector
592 (Nanoliter 2010, World Precision Instruments)
593 controlled by a microsyringe pump controller
594 (Micro4 pump, World Precision Instruments).
595 After retracting the pipette, the wound was
596 rinsed with saline and sutured. The mice were
597 allowed to recover in a heated chamber (37 °C)
598 before returning to the homecage.
599 Postoperative analgesic was applied 6-8 h after
600 the procedure (Temgesic) and 24 h after the
601 procedure (Metacam). The survival time for
602 transduction and expression of the genetic
603 material was 10-15 days.

604

605 In vitro electrophysiology

606 Mice of either sex (>8-week-old) were
607 euthanized with an overdose of pentobarbital
608 (i.p. 100 mg/Kg, Apotekerforeninger), before
609 intracardial perfusion with cutting solution (RT)
610 of the following composition (in mM): 93 choline
611 chloride, 3 KCl, 1.25 NaH_2PO_4 , 30 NaHCO_3 , 20
612 HEPES, 10 glucose, 5 MgCl_2 , 0.5 CaCl_2 , 5 N-
613 acetylcysteine, saturated with 95% O_2 and 5%
614 CO_2 . The brain was extracted and a section
615 containing either PER or wS1 was glued onto
616 the stage of a vibratome (VT1000, Leica) filled
617 with cold (4°C) cutting solution. Slices (300 μm)
618 were transferred to a chamber filled with warm
619 (34°C) cutting solution and incubated for 15
620 minutes. The slices were stored in a different
621 chamber filled with the following solution (in mM)
622 (RT): 92 NaCl, 3 KCl, 1.25 NaH_2PO_4 , 30
623 NaHCO_3 , 20 HEPES, 10 glucose, 5 MgCl_2 , 0.5
624 CaCl_2 , 5 N-acetylcysteine, saturated with 95%
625 O_2 and 5% CO_2 .

626 The slices were placed in a recording chamber
627 of an upright microscope (BW51, Olympus)
628 filled with warm (35°C) recording solution of the
629 following composition: 124 NaCl, 3 KCl, 1.25
630 NaH_2PO_4 , 26 NaHCO_3 , 10 glucose, 1 MgCl_2 ,
631 1.6 CaCl_2 , saturated with 95% O_2 and 5% CO_2 .
632 Characterization of the electrophysiological
633 properties was performed in presence of
634 synaptic blockers: 10 μM DNQXNa₂, 25 μM
635 APV and 10 μM gabazine. wS1 was identified
636 under DIC optics by the presence of
637 characteristic barrels in layer 4. To identify A35
638 under DIC optics, we first identified the lateral
639 entorhinal cortex (LEC) by its characteristic
640 large neurons in layer 2a, and by the clear
641 separation between layer 2a and 2b. A35 was
642 defined as the cortex dorsally located to LEC.
643 Only healthy, fluorophore-expressing neurons

644 were selected for recordings and no other
645 criterion was applied. Whole-cell patch clamp
646 recordings were performed with borosilicate
647 pipettes (Sutter Instruments) with a resistance
648 of 3-7 M Ω filled with the following solution (in
649 mM): 130 K-gluconate, 10 KCl, 10 HEPES, 0.2
650 EGTA, 4 ATP-Mg, 0.3 GTP-Na, 5
651 phosphocreatine- Na_2 , pH 7.3. In all recordings
652 biocytin was added to the pipette solution to
653 recover the layer location of the neuron.
654 Membrane potentials reported were not
655 corrected for a junction potential of -14 mV.
656 Current clamp recordings were performed with
657 a Multiclamp 700B amplifier (Molecular
658 Devices), digitized with a 1550A Digidata
659 (Molecular Devices), interfaced with a personal
660 computer with pClamp 11 (Molecular Devices).
661 Data were sampled at 40 kHz and low-passed
662 filtered at 10 kHz. All recordings were performed
663 from a holding potential of -65 mV. The
664 electrophysiological parameters analyzed were
665 defined as follows:

666 Input resistance (IR, M Ω): resistance measured
667 from Ohm's law from the peak of voltage
668 responses to -25 pA hyperpolarizing current
669 steps.

670 Sag ratio (dimensionless): measured from
671 voltage responses peaking at -90 ± 2 mV to
672 hyperpolarizing current steps. It is defined as the
673 ratio between the voltage at the steady-state
674 response and the peak.

675 Action potential threshold (AP_{thr}, mV):
676 measured from action potentials (AP) evoked
677 near rheobase, and defined as the voltage
678 where the rise of the AP was 20 mV/ms.

679 AP half width (HW, ms): duration of the AP at
680 half amplitude from AP_{thr}, measured from
681 action potentials (AP) evoked near rheobase.

682 Amplitude of the fast afterhyperpolarization
683 (fAHP, mV): measured from AP_{thr}, from action
684 potentials (AP) evoked near rheobase.

685 AHP duration (AHP_{dur}, ms): measure from
686 action potentials (AP) evoked near rheobase,
687 and defined as the time difference between the
688 fAHP and the most depolarized voltage in a 200
689 ms window.

690 Maximal firing frequency (F_{max}, AP/s): maximal
691 firing frequency evoked with 1-s-long
692 depolarizing current steps.

693 Rheobase (Rheo, pA): current level that evoked
694 the first spike during a 1-s-long depolarizing
695 ramp (500 pA/s).

696

697 Histology

698 Mice used for histological analysis were
699 anaesthetized with isoflurane and then
700 euthanized with an overdose of pentobarbital
701 (i.p. 100 mg/Kg, Apotekerforeninger). The mice
702 were intracardially perfused with Ringer solution
703 (0.85% NaCl, 0.025% KCl, 0.02% NaHCO₃)
704 followed by 4% paraformaldehyde (PFA) in
705 phosphate buffer (PB) (pH 7.4). The brain was
706 extracted from the skull and placed in 4% PFA
707 for 3 hours before moving it to a solution
708 containing 15% sucrose in PB overnight. The
709 brains were then stored in 30% sucrose for two
710 days before being sliced at 50 µm with a
711 freezing microtome. Slices were collected in six
712 equally spaced series and placed in tubes
713 containing a solution composed of 30% glycerol,
714 30% ethylene glycol and 40% phosphate buffer
715 saline (PBS). The tissue was stored at -20 °C
716 until used for histology.

717 The slices were washed in PB (3 X 10 minutes)
718 before incubation with blocking solution (0.1%
719 Triton-X, 10% NGS in PB). After blocking the
720 tissue was incubated with the primary antibody
721 for three days at 4 °C in a solution containing:
722 0.1% Triton-X, 1% NGS in PB. After being
723 washed (3 X 1 hour in PB), the tissue was
724 incubated with the secondary antibody
725 overnight at 4 °C. The slices were washed (3 X
726 10 minutes in PB) and mounted on SuperFrost
727 slides (Termo Fisher Scientific) and left to dry
728 overnight. The slides were coverslipped with
729 entellan xylene (Merck Chemicals, Darmstadt,
730 Germany) after being washed in xylene for 15
731 minutes, or with Fluoromount after being
732 washed in distilled water for about 30 seconds.
733 We used the following primary antibodies:
734 Guinea Pig IgG anti-NeuN (1:1000, Sigma
735 Millipore, #ABN90P), Rabbit anti-parvalbumin
736 (1:1000, Swant, #PV-27), Mouse IgG1 anti-
737 parvalbumin (1:1000, Sigma, #P3088), Rat
738 IgG2a anti-RFP (1:1000, Proteintech, #5f8),
739 Chicken IgY anti-GFP (1:1000, Abcam,
740 #ab13970), Rabbit IgG anti-SST (1:1000, BMA
741 Biomedicals), Rabbit IgG anti-VIP (1:1000,
742 Immunostar). We used the following secondary
743 antibodies: Goat anti-guinea pig (IgG H+L) A647
744 (1:500, Invitrogen, #A-21450), Goat anti-Rat
745 (IgG H+L) A-546 (1:500, Invitrogen, #A11081),
746 Goat anti-Rabbit (IgG H+L) A488 (1:500,
747 Invitrogen, #A11008), Goat anti-chicken (IgY
748 H+L) A488 (1:500, Invitrogen, #A11039), Goat
749 anti-rabbit (IgG H+L) A546 (1:500, Invitrogen,

750 #A11010), Goat anti-rabbit (IgG H+L) A635
751 (1:500, Invitrogen, A31576).

752

753 Image acquisition and analysis

754 We used a confocal microscope (Zeiss LSM 880
755 Axiolmager Z2) to image regions of interest
756 (ROIs) to count labeled neurons. Images of PER
757 and wS1 were taken with a x20 air objective with
758 1 Airy unit pinhole size and saved as czi files.
759 We selected 3 to 5 ROIs for each cortical region.
760 ROIs contained the whole cortical area of
761 interest in a given slice. Files containing the
762 ROIs were uploaded in Neurolucida (Micro
763 Bright Field Bioscience) for analysis. We
764 created contours to delineate the layers of the
765 ROIs using the NeuN signal. The PER was
766 divided in area 35 (A35) and area 36 (A36), and
767 delineated according to Beaudin et al. (2013).
768 Symbols for each signal were used to count
769 cells labeled by different markers. We counted
770 all cells contained within the contours. Since we
771 counted slices distanced 300 µm from each
772 other, overcounting in the z axis is not relevant
773 and we did not apply any correction.
774 Quantification of the number of markers in each
775 contour was done in Neurolucida Explorer
776 (Micro Bright Field Bioscience) and exported in
777 excel files. The density of neurons labeled by a
778 marker was measured as the number of labeled
779 neurons in a contour divided by the area of the
780 contour (cells/mm²). The GAD67-eGFP mouse
781 line labelled virtually all PV-INs and VIP-INs, but
782 showed a lower specificity for SST-INs (Figure
783 1 – Figure supplement 1). For this reason, the
784 number of PV-INs, SST-INs and VIP-INs was
785 measured from the immunolabelling for the
786 specific marker, independently of GFP
787 expression. Transcriptomic analysis of the
788 neuronal population of the cortex shows that
789 SST, VIP and PV are expressed exclusively in
790 GABAergic neurons. We excluded from the
791 counting a small population of PV-IR pyramidal
792 neurons in L5B of wS1 (van Brederode et al.,
793 1991). Values are reported as mean ± standard
794 deviation). All graphs were created in excel,
795 images of ROIs were created in Zen lite (Zeiss)
796 and figures in illustrator.

797

798 Statistics

799 Principal component analysis on the densities of
800 different GABAergic neurons across layers of
801 PER and wS1 was performed in Matlab 2021
802 (Mathworks) using the function “pca”. We

803 obtained the explained variance for each
804 principal component, the principal component
805 coefficients, the principal component scores,
806 and the principal component variances.
807 Hierarchical cluster analysis was performed in
808 Matlab 2021 (Mathworks). After normalization
809 with the function “zscore”, we calculated the
810 distance between pairs of objects with the
811 function “pdist” and the method “cityblock”.
812 Objects were grouped according to the
813 calculated distances with the function “linkage”
814 and the method “average”. After calculating the
815 inconsistency index (“inconsistent”), clusters
816 were obtained by using the function “cluster”
817 and the inconsistency index as cutoff. This
818 method identified two clusters.

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836

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840

841 **References**

842

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Figure 1 – Figure supplement 2. A. Bar plot showing the variance explained by each principal component. **B.** Bar plots showing the contribution of each variable (i.e., marker density in a given layer) to the first (left) and second (right) principal component.

Figure 1 – Figure supplement 3. A. Bar plot showing the statistical comparison of the density of PV-IR neurons in the GAD67-EGFP mouse line (n= 5 mice): L1 (F= 2.06, df= 2, p= 0.17, One-way ANOVA); L2/3 (F= 28.54, df= 2, p= 2.75E-5, One-way ANOVA; A35 vs wS1, p= 3.16E-5, Bonferroni correction; A36 vs wS1= 0.0007, Bonferroni correction); L5 (F= 45.5, df= 2, p= 2.5E-6, One-way ANOVA; A35 vs wS1, p= 2.37E-5, Bonferroni correction; A36 vs wS1, p= 0.0001, Bonferroni correction); L6 (F= 8.17, df= 2, p= 0.006, One-way ANOVA; A35 vs wS1, p= 0.011, Bonferroni correction). **B.** Bar plot showing the statistical comparison of the density of SST-IR neurons in the GAD67-EGFP mouse line: L1 (F= 0.21, df= 2, p= 0.82, One-way ANOVA); L2/3 (F= 87.06, df= 2, p= 7.18E-8, One-way ANOVA; A35 vs A36, p= 0.014, Bonferroni correction; A35 vs wS1, p= 6.85E-6, Bonferroni correction; A36 vs wS1, p= 0.0003, Bonferroni correction); L5 (F= 0.53, df= 2, p= 0.6, One-way ANOVA); L6 (F= 12.83, df= 2, p= 0.001, One-way ANOVA; A35 vs A36, p= 0.015, Bonferroni correction; A35 vs wS1, p= 0.004, Bonferroni correction). **C.** Bar plot showing the statistical comparison of the density of GFP-IR/PV-SST neurons in the GAD67-EGFP mouse line: L1 (F= 1.61, df= 2, p= 0.24, One-way ANOVA); L2/3 (F= 51.43, df= 2, p= 1.3E-6, One-way ANOVA; A35 vs wS1, p= 6.04E-6, Bonferroni correction; A36 vs wS1, p= 0.0007, Bonferroni correction); L5 (F= 56.88, df= 2, p= 7.55E-7, One-way ANOVA; A35 vs wS1, p= 1.21E-5, Bonferroni correction; A36 vs wS1, p= 4.16E-5, Bonferroni correction); L6 (F= 38.09, df= 2, p= 6.35E-6, One-way ANOVA; A35 vs A36, p= 0.014, Bonferroni correction; A35 vs wS1, p= 1.99E-5, Bonferroni correction; A36 vs wS1, p= 0.0007, Bonferroni correction).

Figure 2 – Figure 2 supplement 1. A. Representative confocal stack of immunofluorescence for GFP (green) and VIP (red) in wS1 of the GAD67-EGFP mouse line. **B.** Representative confocal stack of immunofluorescence for GFP (green) and VIP (red) in PER of the GAD-EGFP mouse line. **C.** Bar plot showing the statistical comparison of the density of VIP-IR neurons across layers of A35 (red), A36 (yellow) and wS1 (purple) (n= 5 mice): L1 (F= 2.34, df= 2, p= 0.14, One-way ANOVA); L2/3 (F= 0.51, df= 2, p= 0.61, One-way ANOVA); L5 (F= 17.76, df= 2, p= 0.0003, One-way ANOVA; A35 vs wS1, p= 0.0005, Bonferroni correction; A36 vs wS1, p= 0.005, Bonferroni correction); L6 (F= 5.22, df= 2, p= 0.02, One-way ANOVA).

Figure 4 – Figure supplement 1. A-C. Representative confocal stacks of immunofluorescence for GFP (green), PV (white) and dTom (red) in a PV-IRES-Cre mouse injected with the S5E2-dTom virus in wS1. **D.** Bar plot showing the specificity (E2:PV/E2), and efficiency (E2:PV/PV and E2:GFP/GFP) of the S5E2-dTom virus in wS1 (n= 1 mouse). **E-G.** Representative confocal stacks of immunofluorescence for GFP (green), SST (white) and dTom (red) in PV-IRES-Cre mice injected with the S5E2-dTom virus in wS1. **H.** Bar plot showing the percent of neurons expressing dTomato (E2) that co-express SST (E2:SST/E2), and the percent of SST neurons labeled by the S5E2-dTom virus (E2:SST/SST) (n= 1 mouse).