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Interneuron Diversity in the Rat Dentate Gyrus: An Unbiased In Vitro Classification

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1 **Interneuron Diversity in the Rat Dentate Gyrus: An Unbiased *In Vitro* Classification**

2

3 Running title: Dentate Gyrus Interneuron Diversity

4

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17 **Keywords:**

18 dentate gyrus; interneuron; inhibition; GABA; cluster analysis; neuron diversity

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23 **Abstract**

24 Information processing in cortical circuits, including the hippocampus, relies on the dynamic
25 control of neuronal activity by GABAergic interneurons (INs). INs form a heterogenous
26 population with defined types displaying distinct morphological, molecular, and physiological
27 characteristics. In the major input region of the hippocampus, the dentate gyrus (DG), a
28 number of IN types have been described which provide synaptic inhibition to distinct
29 compartments of excitatory principal cells (PrCs) and other INs. In this study, we perform an
30 unbiased classification of GABAergic INs in the DG by combining *in vitro* whole-cell patch-
31 clamp recordings, intracellular labeling, morphological analysis, and supervised cluster
32 analysis to better define IN type diversity in this region. This analysis reveals that DG INs
33 divide into at least 13 distinct morpho-physiological types which reflect the complexity of the
34 local IN network and serves as a basis for further network analyses.

35

1. Introduction

Neuronal activity in cortical circuits requires a tightly controlled and dynamic balance of excitatory and inhibitory synaptic neurotransmission. This balance emerges from and is maintained by local circuit interactions of excitatory glutamatergic PrCs and inhibitory GABAergic INs (Booker and Vida 2018; Freund and Buzsáki 1996; Pelkey et al. 2017). In contrast to the mostly homogenous populations of PrCs, INs are highly diverse with respect to their morpho-physiological characteristics. As such, distinct IN types are presumed to play divergent roles in the neural circuitry (Bartos et al. 2007; Mott et al. 1997; Somogyi and Klausberger 2005; Vida et al. 2006). This functional diversity of INs is particularly important in their control of PrC excitability by feed-forward and feed-back inhibition, leading to temporal coordination of ensemble activity and network oscillations required for integration of synaptic information (Buzsáki 1984; Gloveli et al. 2005).

In mammals, the hippocampal formation encodes egocentric spatial and contextual information and acts as an integrator for multimodal streams leading to memory trace formation (Andersen et al. 1973; Morris et al. 1982; O'Keefe and Dostrovsky 1971). Spatial information arrives at the hippocampus from the entorhinal cortex (EC) through the dentate gyrus (DG), which transforms the dense firing pattern of the EC into a sparse output for the downstream Cornu Ammonis (first CA3 and then CA1 area) (Hainmueller and Bartos 2020). This sparsification and orthogonalization of cortical code in the DG depends heavily on the activities of diverse INs innervating the two major PrC types, dentate granule cells (DGCs) and hilar mossy cells (MCs) (Amaral et al. 2007). While previous studies described several distinct IN types in the rat DG (Armstrong et al. 2012; Ceranik et al. 1997; Han et al. 1993; Mott et al. 1997; Seress and Ribak 1983; Sik et al. 1997), a systematic and unbiased analysis of their diversity has not been performed.

Prior studies on DG INs have considered the morphology of the neurons as a primary classifier. Their physiological diversity has been almost solely defined by their action potential (AP) discharge properties, subdividing INs into fast-, regular-, or slow-spiking cells (Armstrong et al. 2011; Bartos et al. 2007; Gloveli et al. 2005; Sullivan et al. 2011; Vida et al. 2006; Ylinen et al. 1995). The first systematic analysis, integrating morphological and physiological properties to classify DG IN subtypes was performed in the mouse by Hosp et al. 2014 and revealed at least 5 IN classes. Previous estimates in the rat suggest a greater diversity (Booker and Vida 2018), but a comprehensive determination of IN heterogeneity in the DG has yet to be performed.

Therefore, in the present study, we perform an unbiased, multivariate statistical approach to define DG IN diversity based on their morpho-physiological properties from *ex vivo* brain

72 tissue. Using a transgenic rat expressing the yellow fluorescent protein (YFP, Venus-variant)
73 under the vesicular GABA transporter (vGAT) promoter, we targeted INs in a systematic and
74 quasi-random manner for whole-cell patch-clamp recordings followed by morphological
75 reconstruction and analysis. We then performed a post-hoc cluster-analytical classification of
76 IN types based on measured morpho-physiological characteristics. Our results revealed a
77 higher IN diversity than previously reported, with IN types aligned to major synaptic pathways
78 within the DG local circuit with implications for circuit function.

79

80 **2. Materials and Methods**

81 **2.1. Acute slice preparation**

82 All experiments and animal procedures were performed in accordance with local (LaGeSo,
83 Berlin, T 0215/11) and national guidelines (German Animal Welfare Act). To facilitate efficient
84 and unbiased sampling, we used acute brain slices obtained from 18-26 days-old Wistar rats,
85 expressing a modified YFP (Venus variant) in forebrain INs under the vGAT promoter
86 (Uematsu et al. 2008).

87 *In vitro* hippocampal slices were prepared as previously described (Booker et al. 2014; Degro
88 et al. 2015). Briefly, rats were anesthetized with isoflurane, decapitated and the brains rapidly
89 removed into ice-cold carbogenated (95% O_2/5% CO_2) sucrose-based artificial cerebrospinal
90 fluid (sucrose-ACSF; in mM: 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 25 Glucose, 75
91 Sucrose, 1 Na_2-Pyruvate, 1 Na_2-Ascorbate, 7 $MgCl_2$, 0.5 $CaCl_2$). Transverse hippocampal
92 slices (300 µm nominal thickness) were cut from the ventro-medial hippocampus on an
93 oscillating blade vibratome (VT1200s, Leica, Germany) in ice-cold sucrose-ACSF. Slices
94 were transferred to submerged storage chambers containing sucrose-ACSF warmed to 35°C
95 for 30 min to allow for recovery. Slices were then stored at room temperature (20°C) in the
96 same solution until recording.

97 **2.2. Whole-cell patch-clamp recordings**

98 For electrophysiological recordings, slices were transferred to a submerged recording
99 chamber and perfused with carbogenated, normal ACSF (in mM: 125 NaCl, 2.5 KCl, 25
100 $NaHCO_3$, 1.25 NaH_2PO_4, 25 Glucose, 1 Na_2-Pyruvate, 1 Na_2-Ascorbate, 1 $MgCl_2$, 2 $CaCl_2$).
101 ACSF was flowed at a rate of 10-12 ml/min (Hájos et al. 2009) at a near physiological
102 temperature (32 ± 0.4°C) by an in-line heater (SuperTech, Switzerland). Slices were
103 visualized using an upright microscope (BX-50, Olympus, Hamburg, Germany) equipped with
104 a 40x water immersion objective lens (N.A. 0.8) and epifluorescent illumination. YFP-positive
105 cells were selected for recordings from all layers of the DG (ML, molecular layer; GCL,
106 granule cell layer; polymorphic layer, hilus). YFP-negative DGCs and MCs were recorded as

107 reference populations from the GCL and the hilus, respectively. Recording pipettes were
108 pulled from borosilicate glass capillaries (2 mm outer/1mm inner diameter, Hilgenberg,
109 Germany) on a horizontal pipette puller (P-97, Sutter Instruments, CA, USA) and filled with
110 intracellular solution (in mM: 130 K-gluconate, 10 KCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES, 2 Na_2-
111 ATP, 0.3 Na_2-GTP, 1 Na_2-Creatinine and 0.1% Biocytin; 290-310 mOsm). The resistance of
112 the filled pipettes was 3-5 MΩ. Whole-cell patch-clamp recordings were performed using a
113 MultiClamp 700B amplifier (Molecular Devices, USA) with all signals filtered online at 10 kHz
114 using the built in 4-pole Bessel filter (Axon Instruments, CA, USA), digitized and recorded at
115 20 kHz (NI USB-6212 BNC, National Instruments, Berkshire, UK) using WinWCP software
116 (courtesy of John Dempster, Strathclyde University, Glasgow, UK). Data was analyzed offline
117 using the open source Stimfit software package (Guzman et al. 2014; http://www.stimfit.org).

118 **2.3. Measurement of intrinsic physiological properties**

119 Physiological properties of neurons were entirely analyzed in the whole-cell configuration.
120 Resting membrane potential (V_m) was taken at baseline zero-current level in current-clamp
121 mode at the start of the recordings and further physiological characterization was performed
122 on the basis of voltage responses to a family of hyper- to depolarizing current pulses (50 pA,
123 500 ms duration) ranging from -250 pA to 250 pA (in a subset of neurons followed by a 500
124 pA pulse); liquid junction potential was not corrected. AP properties and threshold were
125 analyzed on the basis of the first AP at rheobase (average of the measured values from 3
126 traces), triggered by a series of small depolarizing current pulses applied to the V_m (10 pA
127 increase, 500 ms duration). AP threshold was determined as the voltage where the rate of
128 rise first exceeded 20 mV/ms. Fast and medium after-hyperpolarization (AHP) were defined
129 as the first and second negative peaks in voltage following the AP measured from threshold.
130 AP discharge frequency (APs overshooting 0 mV) was measured over the full 500 ms trace
131 for depolarizing stimuli. AP adaptation was measured from a train of APs evoked at 250 pA
132 and expressed as the ratio of the first and last interspike interval (ISI). Voltage sag was
133 measured in response to -250 pA current pulses as the difference between the peak and
134 steady state voltage response, expressed as % of peak (3-trace average). Membrane time-
135 constant was calculated in current-clamp mode by fitting a mono-exponential function to the
136 decay of the average response to small hyperpolarizing current pulses (-10 pA, 500 ms
137 duration, 30-trace average). Finally, input resistance (R_{in}) and membrane capacitance (C_m)
138 were assessed in voltage-clamp mode at -60 mV from the average response to small voltage
139 steps (-10 mV, 500 ms duration, 10-trace average). R_{in} was calculated from the steady-state
140 current at the end of the -10 mV pulse from preceding baseline. C_m was derived by fitting a
141 biexponential function to the decay of the capacitive current induced by the pulse following
142 the equation:

143 $$C_m = \tau(w) * (1/R_s + 1/R_{in})$$

144 Where $\tau(w)$ is the weighted time-constant and R_s the series resistance. A detailed overview
145 and description of all assessed physiological parameters are given in the Supporting
146 Information Table 1.

147

148 **2.4. Visualization, imaging, reconstruction and morphological analysis**

149 Morphological characterization of recorded neurons was performed as previously described
150 (Degro et al. 2015). Briefly, following completion of recording, an outside-out patch
151 configuration was obtained and slices were fixed immediately with 4% paraformaldehyde
152 (PFA) in 0.1 M phosphate buffer (PB) overnight at 4ºC. Slices were then rinsed repeatedly in
153 PB prior to incubation with Alexa Fluor 647-conjugated streptavidin (1:1000, Invitrogen,
154 Dunfermline, UK), diluted in PB containing 0.1% Triton X-100 and 0.05% sodium azide
155 (NaN_3), overnight at 4ºC. Slices were finally mounted on glass slides, containing a 300 µm
156 thick agar spacer, with a polymerizing mounting medium (Fluoromount-G, Southern Biotech,
157 AL, USA) and cover-slipped.

158 All recorded cells were imaged on a laser scanning confocal microscope (FluoView 1000,
159 Olympus) with either 20x (NA 0.75) or oil-immersion 60x (NA 1.3) objective lenses. For 3D
160 reconstructions of the imaged cells, image stacks were collected along the z-axis of the cells
161 (0.5 or 1 µm steps, 4 µs pixel dwell time, 1024 x 1024 or 2048 x 2048 resolution).
162 Neighboring z-series images were then stitched using the FIJI software package
163 (http://fiji.org) and reconstructions of the labeled neurons were made with the semi-automatic
164 Simple Neurite Tracer plug-in for FIJI (Longair et al. 2011). Traces of the neuronal structure
165 were then reformatted in ´.swc´ files for post-hoc corrections: z-axis slice shrinkage was
166 compensated by calculating and applying a correction factor representing the quotient of the
167 original slice thickness (300 µm) and the imaged thickness of the specimen. Segmented
168 neuronal reconstructions were then smoothed in NEURON (Hines and Carnevale 1997)
169 using a Gaussian spatial filter (3-point window, single run in the x/y-plane and 10 iterations
170 for values along the z-axis; customized hoc script, Bolduan et al. 2020) to reduce imaging
171 artefacts. Finally, morphometric parameters were analyzed with 1) the open source L-
172 measure software package (Scorcioni et al. 2008; branch order, branch pathlength,
173 bifurcation angle, no. of dendritic stems) 2) R macroinstructions (The R Project for Statistical
174 Computing; https://www.r-project.org; customized R scripts: axon density parameters) and 3)
175 the NEURON simulation program (Hines and Carnevale 1997; customized hoc scripts: axon
176 and dendritic polarity). Sholl analysis parameters and compartment specific distribution of the
177 axonal and dendritic arbor were assessed in FIJI by using the Sholl Analysis plug-in and the

178 Segmentation Editor plug-in, respectively, by labeling the region of interest (ROI) and
179 calculating the relative length of the trajectories that fall within the ROI. A detailed overview
180 and description of all assessed morphological parameters are given in the Supporting
181 Information Table 2.

182 **2.5. Immunohistochemistry**

183 To reveal differences in neurochemical marker expression of recorded neurons, we
184 performed immunofluorescent labeling. Slices were first rinsed in 25 mM PB containing 0.9%
185 NaCl (PBS) and then blocked in a solution containing 10% Normal Goat Serum, 0.3% or 1%
186 Triton X-100 and 0.05% NaN_3, diluted in PBS, for 60 min at room temperature. Slices were
187 then transferred to a mixture of primary antibodies (PBS containing 5% Normal Goat Serum,
188 0.3% Triton X-100 and 0.05% NaN_3) for 120 min (room temperature) before washing in PBS
189 and subsequently incubated in a secondary antibody solution (Alexa Fluor 405/546 or 594,
190 1:1000, Invitrogen, diluted in PBS containing 3% Normal Goat Serum, 0.1% Triton X-100 and
191 0.05% NaN_3, overnight, 4°C). Finally, slices were rinsed in PBS, desalted with PB and
192 mounted on glass slides. The presence of immunofluorescence was tested by confocal
193 imaging over the soma and proximal dendrites.

194 Selection of the different primary antibodies was based on previously described
195 neurochemical distributions in the DG (Freund and Buzsáki 1996; Hosp et al. 2014) and is
196 shown in Table 1. When possible, neurons were assessed for 2-3 neurochemical markers
197 simultaneously.

198 **2.6. Kernel principal component (KPCA) and cluster analysis**

199 Morpho-physiological cluster analysis was performed with a total of 87 fully reconstructed
200 neurons (80 INs, 4 DGCs and 3 MCs) on the basis of 53 parameters (38 morphological and
201 15 physiological). A Kaiser-Meyer-Olkin (KMO) index of 0.53 and a significant Bartlett´s test
202 of sphericity ($p<0.001$) indicated factorization sampling adequacy of the combined morpho-
203 physiological data (morphological dataset: KMO index: 0.52, Bartlett´s test of sphericity:
204 $p<0.001$; physiological dataset: KMO index: 0.62, Bartlett´s test of sphericity: $p<0.001$;
205 Bartlett 1950, Supporting Information Table 12) using a KMO index cut-off >0.5 (Field et al.
206 2000; Kaiser 1974). In view of the non-linear structure of the given dataset, a KPCA
207 (Karatzoglou et al. 2004; Schölkopf et al. 1998) was then applied prior to cluster analysis.
208 KPCA was performed, based on normalized parameters, by calculating a kernel matrix using
209 the following radial basis function (RBF) kernel

210 $$k(x,x')= \exp(- \sigma \|x - x'\|^2)$$

211 followed by a centering of the kernel matrix and principal component calculation (´kpca´
212 function, kernlab package, R). The inverse kernel width, σ, for the RBF kernel function was

213 calculated using the ´sigest´ function (kernlab package, R) and set to the 0.5 quantile value.
214 The scree-test (defined as *k*-1) was then used to select the principal components to retain (*k*
215 is defined as the kink-point in a principal component/eigenvalue plot; Bacher et al. 2010;
216 Cattell 1966). Finally, data was projected onto the extracted principal components, from
217 which a deterministic hierarchical-agglomerative cluster analysis, following Ward's minimum
218 variance method (HCA Ward) was performed (´hclust´ function, stats package, R). As such,
219 the proximity measure that describes the distance between 2 clusters equated to the squared
220 Euclidean distance (*see* Supporting Information Tables 9-11). The cluster results were
221 represented by a classic dendrogram, with the optimal number of clusters calculated using
222 the inverse scree-plot where the number of clusters to retain equals *k*, when *k* is the kink-
223 point in a total number of clusters/merging level plot ("elbow-criterion", Bacher et al. 2010).
224 Cluster analyses of morphological and physiological characteristics alone were performed
225 identical to the above described algorithm. Additional clustering methods (k-means and
226 divisive analysis, DIANA) that are reported in the Supporting Information were performed
227 using the ´kmeans´ function (stats package, R) and the ´diana´ function (cluster package, R).
228 Full details of all R functions used are outlined in the Supporting Information.

229 **2.7. Chemicals and pharmacological tools**

230 All chemicals were obtained from either Sigma Aldrich (Munich, Germany) or Carl Roth
231 (Karlsruhe, Germany). Biocytin was obtained from Life Technologies (Dunfermline, UK).
232 Working solutions (ACSF) were prepared fresh on each experimental day.

233 **2.8. Statistical analysis**

234 Cluster analyses were performed with R (Ripley 2001; The R Project for Statistical
235 Computing; https://www.r-project.org) and plots of data were generated with R and
236 GraphPad Prism 9.0.0 (GraphPad Software, CA, USA). Statistical significance was assumed
237 if the resulting *p*-value was <0.05. This was assessed using either a two-way ANOVA or a
238 Kruskal-Wallis test. Data is shown as mean ± SD throughout.

239

240 **3. Results**

241 **3.1. Identification of INs and PrCs in the DG**

242 To investigate the diversity of DG INs, we performed whole-cell patch-clamp recordings
243 combined with intracellular biocytin labeling in acute hippocampal slices from vGAT-YFP rats
244 (Uematsu et al. 2008). These rats expressed YFP under the vGAT promoter that enabled an
245 efficient and systematic sampling of INs. In slices from these rats, YFP-positive neurons

246 were scattered in all layers of the DG, with the highest abundance found in the polymorphic
247 hilus region adjacent to the GCL (Fig. 1A).

248 Recorded INs displayed a variety of heterogeneous electrophysiological properties, ranging
249 from high-frequency, non-accommodating to regular-spiking accommodating AP discharge
250 patterns (Fig. 1B-D, insets). Visualization of intracellularly-labeled cells also revealed
251 divergent morphologies with respect to dendrite and axon distributions (Fig. 1B-D).
252 Depending on soma localization, the dendrites of these INs displayed horizontal, vertical,
253 pyramidal-like, or multipolar morphologies, which were typically non-spiny or occasionally
254 sparsely spiny. The axon of INs typically emerged from the soma or a proximal dendrite
255 forming a dense local axon. This axon often showed a laminar distribution – consistent with a
256 compartment specific synaptic output within the DG. Visual inspection of the INs suggested
257 that this sample included cells with previously described morpho-physiological properties: for
258 example perisomatic inhibitory fast-spiking basket cells (BC, Fig. 1B) and dendrite-targeting
259 INs, such as hilar perforant pathway associated (HIPP) cells (Fig. 1C). Post-hoc
260 immunolabeling for neurochemical IN markers demonstrated the differential presence of
261 parvalbumin (PV) and somatostatin (SST) in these INs, respectively, consistent with their
262 putative identity (Fig. 1B and C, insets). In contrast to the previously described types, many
263 INs displayed divergent morpho-physiological properties with varied neurochemical
264 expression such as pro-cholecystokinin (pCCK, Fig. 1D), indicating that the previous
265 classification of DG INs is likely incomplete.

266 YFP-negative neurons formed two major populations: first with small, round somata, densely
267 packed in the GCL and second with large somata localized to the hilus. Recordings from
268 YFP-negative cells located in the GCL displayed electrophysiological characteristics of DGCs
269 including accommodating discharge patterns and hyperpolarized membrane potentials.
270 Visualization revealed typical DGC morphology consisting of 3-6 monopolar, densely spiny
271 dendrites that fanned out into the ML covering all sublayers. DGC axons uniformly emerged
272 from the opposite pole of the soma and projected through the hilus and towards the CA3
273 (Fig. 1E). Recordings from YFP-negative neurons in the hilus showed morpho-physiological
274 features consistent with MCs, including a multipolar somato-dendritic domain confined to the
275 hilus, densely covered with simple and large complex spines. MCs axonal projections were
276 restricted to the hilus and the inner ML (iML, Fig. 1F).

277 To define the diversity of DG INs in an unbiased manner, we next performed a detailed
278 morphological and electrophysiological analysis of the recorded neurons combined with an
279 unbiased hierarchical clustering. We performed this analysis with complete morphological
280 and electrophysiological characterization on a total of 80 YFP-positive INs and 7 YFP-
281 negative PrCs.

282 **3.2. Cluster analysis of DG IN morphotypes**

283 A major criterion in previous classifications of IN and PrC types was their morphology, in
284 particular the laminar distribution of axon and dendrites (Booker and Vida 2018; Freund and
285 Buzsáki 1996; Han et al. 1993; Pelkey et al. 2017). As such, we first performed a hierarchical
286 cluster analysis of morphological parameters using Ward's minimum variance method (Fig.
287 2). All neurons were 3-dimensionally reconstructed, from which 38 morphological parameters
288 were derived (axon: 20, dendrites: 16, soma: 2, Supporting Information Table 2). Based on
289 these parameters, we first performed a KPCA to achieve a general dimensional reduction. By
290 applying the scree-test (Cattell 1966), we included the first 7 principal components in our
291 HCA Ward which together contributed 73% of the morphological variance (Fig. 2A).

292 To determine the number of neuronal clusters, we next applied the “elbow”-criterion (Bacher
293 et al. 2010; Fig. 2B, inset) and identified 14 distinct morphological clusters, 12 of which
294 comprised different YFP-positive IN morphotypes. The two main branches of the dendrogram
295 were generally comprised of INs with soma localization either restricted to the ML or the
296 hilus/GCL, associated with the largest squared Euclidean distance (Fig. 2B). The first branch
297 comprised ML INs that formed 4 of the 12 clusters (M1-4). The second branch included 5
298 clusters of INs preferentially localized to the hilus (M8-12) and 3 clusters with somata within,
299 or adjacent to the GCL (M5-M7). The individual clusters in both branches showed further
300 divergence with respect to their axonal and dendritic distributions (Fig. 2B and 2C). Indeed,
301 in the ML IN clusters we observed both previously described and novel morphotypes. While
302 most clusters with somata in the ML displayed prominent axon alignment within this layer,
303 differences existed in the pattern of branching and the precise laminar distribution of these
304 collaterals. Notably, M3 neurons (N=5) had a highly branched, dense, focal axon and
305 dendrites largely restricted to the outer ML (oML), comparable to those of neurogliaform cells
306 (NGFCs), but also projected into the subiculum/CA1. By contrast, M2 cells (N=8) had somata
307 and dendrites confined to the ML and an axon which showed a broad horizontal distribution
308 in the middle ML (mML) and to a lesser extent in the oML and iML, characteristics
309 reminiscent of ML perforant pathway associated (MOPP) neurons.

310 In addition, we identified 3 clusters of ML morphotypes that were not previously described:
311 M1 cells (N=5) revealed an axonal distribution comparable to that of the MOPP like (M2)
312 cluster, but also projected across the hippocampal fissure into the subiculum/CA1. In
313 contrast to M2 INs, their dendrites were mainly restricted to the mML and oML. M4 neurons
314 (N=9) were characterized by a somatodendritic localization in the oML direct adjacent to the
315 hippocampal fissure and by a substantial axonal projection into the subiculum/CA1 (% of
316 axon: 20.7 ± 15.7). Unlike NGFC like (M3) INs, M4 neurons displayed a larger horizontal
317 extent of their axonal arbor with a reduced perisomatic axonal density (% of axon: 13.5 ± 3.7

318 vs. 25.6 ± 7.7, Supporting Information Table 3 and Supporting Information Figure 7). In
319 contrast, the M5 cluster (N=6) had somata and axons restricted to the iML, but with dendrites
320 spanning all layers.

321 In the second branch of the dendrogram, we identified specific clusters that had
322 morphologies resembling previously described IN types (Hosp et al. 2014). For example, the
323 M6 (N=4) and M7 (N=5) clusters had somata localized in or adjacent to the GCL with
324 dendrites spanning all layers. Their dense axonal arbors were largely confined to the GCL,
325 corresponding to putative axo-axonic (AACs) and basket cells (BCs). The M8 cluster (N=3)
326 was characterized by neurons with spiny dendrites restricted to the hilus and axons localized
327 to the outer two thirds of the ML, bearing strong resemblance to hilar perforant pathway
328 associated (HIPP) cells. M10 neurons (N=9) showed a preferential axonal projection to the
329 iML, a characteristic similar to hilar commissural-associational pathway associated (HICAP)
330 cells. However, in contrast to the original description of HICAP neurons, the majority of the
331 M10 axon collaterals were confined to the hilus. M11 cells (N=8) featured an axonal arbor
332 that covered all layers of the ML, consistent with total molecular layer (TML) cells. In addition
333 to these previously described hilar IN morphotypes, we identified 2 IN clusters within this
334 group which had markedly different morphologies. Specifically, M12 (N=13) had a dendritic
335 distribution covering all layers of the DG, but possessed an axon that preferentially ramified
336 in the mML (Fig. 2C). M9 (N=5) displayed a dendritic domain restricted to the hilus, but had
337 an axon that appeared bistratified, targeting both the outer two thirds of the ML and the hilus.

338 Importantly, YFP-negative PrCs, included in the analysis for reference, clearly segregated
339 from the above IN clusters, with both DGCs and MCs clustering into two distinct
340 morphotypes (Fig. 2B). Comparison of the given HCA Ward with two different cluster
341 methods (divisive hierarchical clustering, DIANA, and partional clustering, k-means)
342 generally showed a strong overlap of the revealed cluster constitutions, most distinct for the
343 k-means clustering (89% overlap, Supporting Information Figure 4). A summary of the entire
344 morphological dataset of each morphological cluster identified is presented in Supporting
345 Information Figure 1 and 7 and in Supporting Information Tables 3 and 4.

346 **3.3. Cluster analysis of physiological properties of DG INs**

347 INs also show a variety of physiological properties (Scharfman 1995) which have been
348 previously used as a key dissector of types (Hosp et al. 2014), serving as a partial classifier
349 for their wider diversity (Gouwens et al. 2020). As such, we next performed a cluster analysis
350 based on physiological properties of the recorded neurons (5 intrinsic membrane properties
351 and 10 AP properties, Supporting Information Table 1). After applying the scree-test on the
352 KPCA results, we identified 5 principal components that contributed 81% of observed
353 variance (Fig. 3A). These 5 principal components were retained and included in our HCA

354 Ward, which resulted in a hierarchical classification subdividing the recorded neurons into 8
355 physiological clusters ("elbow-criterion") with distinct electrophysiological properties (P1-P8;
356 Fig. 3B and C). Comparison of the result obtained using Ward's method with the other two
357 clustering methods displayed a good correlation of the proposed cluster constitutions (k-
358 means: 68% overlap; DIANA: 74% overlap; Supporting Information Figure 5), however less
359 pronounced than the morphological clustering.

360 Overall, the physiological clustering separated physiological (P)-types, including PrCs (P4
361 and P6) and INs (P1-3, P5, P7 and P8). This is reflected by diverse patterns of AP discharge
362 from IN clusters and PrCs. Indeed, within the P-types, P1 (N=11) represented typical fast-
363 spiking INs, with small amplitude, rapid APs and large and fast AHPs (Fig. 4A, D and E). The
364 remaining IN P-types had trains of APs with higher frequency discharge than PrCs, but
365 displayed passive and active properties that were highly heterogeneous between clusters,
366 thus reflecting cell-type specific diversity (Fig. 4B-E). A further key feature contributing to
367 physiological clustering related to AP kinetics, as such we then compared these properties
368 between P-types, both as the voltage response (Fig. 4D) and as the first-derivative of the
369 voltage (phase plots, Fig. 4E). As expected from the diverse AP discharge patterns seen, the
370 AP kinetics of P-types were distinct, with large-amplitude, fast APs in P4 (N=4),
371 corresponding to DGCs, and small-amplitude, fast APs in P1, corresponding to classic "fast-
372 spiking" cells. The remaining INs and MCs displayed APs of similar amplitude, but with a
373 spectrum of kinetic properties (Figure 4D and E). Based on previous observations (Hosp et
374 al. 2014), the passive properties of DG INs are also divergent. To confirm this, we plotted the
375 mean current-voltage relationship of each identified P-type in response to hyperpolarizing
376 current pulses (Fig. 4B). This agreed with both the spike discharge and AP kinetic properties,
377 revealing a high degree of diversity in voltage response, ranging from low-resistance neurons
378 (P1, P3, P6) to those with much higher voltage responsivity (P5, P7, P8; Fig. 4B). In line with
379 the P-type classification identified distinct types of DG INs, phase plots of AP kinetics for
380 each cluster displayed homogeneous AP kinetics (P2, P6-7), while others displayed high
381 variability (P1, P3-5 and P8; Fig. 4E). This was exemplified by several DGCs inappropriately
382 being assigned to otherwise mostly IN types (P5), likely reflecting the more variable nature of
383 electrophysiological recordings compared to anatomy alone.

384 This data shows that, while our physiological cluster analysis of DG neurons alone revealed
385 that particular P-classes gave rise to distinct functional types, the total number of clusters
386 identified based on electrical properties alone was lower than for the morphological
387 classification and showed greater heterogeneity within type. A summary of the entire
388 physiological dataset of each physiological cluster identified is presented in Supporting
389 Information Figure 8 and in the Supporting Information Tables 5 and 6.

390 **3.4. Correlation of morphological and physiological derived IN cluster**

391 We next correlated the single cell constitutions originated from the morphological and
392 physiological parameter clustering to detect potential overlap and divergence between these
393 two cluster results. Comparison of the separate cluster analyses (Fig. 4F) showed that while
394 some morphotypes possessed distinct physiological properties, others did not. In particular,
395 the M6 and M7 morphotype showed a high convergence with the P1 cluster reflecting the
396 fast-spiking characteristic of these perisomatic-inhibitory INs. Likewise, the M8 morphotype
397 showed a marked overlap with the P8 cluster. Importantly, P4 and P6 generally showed a
398 good overlap with DGCs and MCs respectively, confirming the validity of their classification.
399 However, most of the other morphotypes consisted of a variety of physiological types. A key
400 distinguishing feature between morphotypes related to the P3 cluster, which generally
401 overlapped with ML INs (M1 - M4). This ML IN P-type generally had a V_m that was more
402 hyperpolarized (-72.2 ± 6.0 mV) than P-types associated with hilus INs (-60.9 ± 7.2 mV).
403 Finally, within hilus IN clusters, there was loose overlap of morpho-physiological features,
404 specifically M5 to P2, M11 to P8 and M12 to P2/P5. This analysis confirms that morphology
405 and physiology alone are not sufficient to define distinct DG IN types, as such a combined
406 approach is required.

407 **3.5. Combined morpho-physiological clustering reveals greater diversity of DG INs**

408 As neither morphological nor physiological properties alone gave rise to a uniformly
409 convergent IN classification, we next performed a combined morpho-physiological clustering
410 which included all previously used descriptors. Performing KPCA followed by the scree-test,
411 we identified 9 principal components that constituted 74% of observed variance (Fig. 5A).
412 Based on the principal components of this combined analysis, our HCA Ward revealed 15
413 distinct clusters of neurons following implementation of the “elbow-criterion” (Fig. 5B, inset),
414 which reflected the high IN diversity in the DG whilst also separating identified DGCs and
415 MCs (Fig. 5B) and was largely consistent with different clustering methods (k-means: 77%
416 overlap; DIANA: 76% overlap, Supporting Information Figure 6). The relative magnitude of
417 each parameter in relation to the identified morpho-physiological clusters is shown in Fig 5C
418 with morpho-physiological parameters ordered by an independent Ward clustering. This
419 combined approach resembled morphological clustering alone (Fig. 2B), albeit with a number
420 previously classified IN types.

421 BCs (N=5) and AACs (N=5) contributed two separate clusters, clearly representing two
422 discrete morpho-physiological IN types within the DG. Of the clustered BCs, all displayed the
423 typical dense axonal arbor that ramified heavily around DGC somata and multipolar
424 dendrites spanning the ML and extending deep into the hilus. Their physiological
425 characteristics comprised high-frequency trains of low-amplitude, fast APs in response to

426 depolarizing current pulses, which were followed by large and fast AHPs. Typically, their V_m
427 was depolarized, with low R_{in} and rapid membrane time constants. By contrast, the AAC
428 cluster displayed a similar dendritic distribution, but with somata predominantly found at the
429 GCL/ML border and with cartridge-like axon collaterals oriented perpendicularly to the
430 GCL/hilus border. Despite producing high-frequency trains of APs in response to
431 depolarizing stimuli, AACs had slower AP kinetics, larger R_{in} and lower C_m compared to BCs,
432 which were the major separating physiological criteria (Table 3 and 4). Nevertheless, BC and
433 AAC clusters clearly segregated from the remaining hilus INs as emphasized by a large
434 squared Euclidean distance of proportional 43.3%. This additionally highlights that BCs and
435 AACs may occupy a unique niche in the DG network.

436 Other previously identified IN types included: (1) HIPP cells (N=3), which had densely spiny
437 somato-dendritic domains localized to the hilus with axon projections to the outer two thirds
438 of the ML. HIPP cells showed a regular-spiking phenotype, with minimally adapting, large
439 amplitude APs and fast AHPs. Their V_m was depolarized, with relatively high R_{in} and long
440 membrane time constants. (2) HICAP cells (N=7) with sparsely spiny dendrites spanning all
441 layers of the DG and a characteristic axonal innervation of the iML. Hyperpolarized V_m, small
442 voltage sag - indicating a low I_h- and highly adaptive trains of slow APs typified HICAP
443 physiology. (3) TML cells (N=9) which had hilar somata giving rise to multipolar, aspiny or
444 sparsely spiny dendrites and axons spanning all layers of the DG. TML AP trains showed
445 strong adaptation with relatively fast AP kinetics, despite high R_{in} and hyperpolarized V_m. (4)
446 MOPP cells (N=11), with somata and aspiny dendrites restricted to the ML and an axon that
447 predominantly ramified in the outer two thirds of the ML. MOPP cells possessed a
448 pronounced hyperpolarized V_m, with low C_m and rapid membrane time constants. MOPP AP
449 discharge showed a regular-spiking nature, no adaptation and large, fast AHPs. 5) NGFCs
450 (N=3), characterized by small somata located in the oML, giving rise to multiple short,
451 profusely branching, aspiny dendrites that were locally restricted. The axon of NGFCs was
452 extremely dense, and locally restricted. NGFCs possessed hyperpolarized V_m, short
453 membrane time constants and the lowest C_m of any IN cluster – well reflecting their compact
454 morphology. Their AP discharge pattern was uniformly regular-spiking, with no adaptation
455 and small AP amplitudes.

456 Beyond these previously described types, the combined cluster analysis also revealed a
457 number of novel IN types, which we have named based on the previously used nomenclature
458 for DG INs (Freund and Buzsáki 1996; Han et al. 1993). These new types are as follows:

459 **S**ubiculum **P**rojecting I (**SP,** cluster M1, M3-M4; cluster P3) cells: SP I cells (N=7) were
460 found in the outer two thirds of the ML, with broad preference to the mML. They displayed
461 predominantly monopolar aspiny dendrites with branches mostly found in the mML

462 (30.1 ± 12.7% of dendrites) and oML (61.2 ± 12.2% of dendrites), but occasionally crossing
463 the hippocampal fissure into the subiculum/CA1 (5.1 ± 4.3% of dendrites). Their multipolar
464 axon projections covered all layers of the ML, with the majority restricted to the mML
465 (28.2 ± 11.5% of axon) and oML (45.3 ± 19.7% of axon). Characteristically, their axon
466 showed numerous collaterals in the subiculum/CA1 region (19.0 ± 11.9% of axon). SP I cells
467 had a hyperpolarized V_m (-76.1 ± 3.7 mV), a relatively low R_{in} (153.5 ± 35.6 MΩ) and a rapid
468 membrane time constant (8.9 ± 2.0 ms). They fired small and slow APs (AP amplitude:
469 49.8 ± 3.2 mV; half-height duration: 0.88 ± 0.10 ms) with low voltage thresholds (-28.9 ± 3.7
470 mV).

471 **S**ubiculum **P**rojecting II (**SP,** cluster M4; cluster P3) cells: Similar to SP I cells, the SP II type
472 (N=6) was characterized by its prominent axonal projection into the subiculum/CA1.
473 However, these INs had relatively small somata located to the oML in the immediate vicinity
474 to the hippocampal fissure, which gave rise to radial, aspiny or sparsely spiny dendrites
475 primarily in the oML (82.6 ± 15.5% of dendrites), which also crossed the hippocampal fissure
476 into the subiculum/CA1 (10.9 ± 11.0% of dendrites). In contrast to SP I neurons, SP II cells
477 possessed a predominant horizontally-oriented axon which extended over both the supra-
478 and infra-pyramidal blades of the DG (max. axon radius: 576.7 ± 115.5 µm) and was present
479 in the oML (64.6 ± 20.3% of axon), subiculum/CA1 (19.7 ± 16.6% of axon) and to a lesser
480 extent in the mML (14.9 ± 11.7% of axon). SP II cells displayed a strongly hyperpolarized V_m
481 (-73.3 ± 2.1 mV), short membrane time constants (10.7 ± 2.3 ms) and a low C_m (46.5 ± 9.1
482 pF). Discharge properties of the SP II type revealed non-adapting (ISI 1st/last: 1.04 ± 0.09)
483 trains of low amplitude and slow APs (AP amplitude: 51.0 ± 3.1 mV; half-height duration:
484 0.83 ± 0.06 ms).

485 **Mo**lecular Layer **C**ommissural-**A**ssociational **P**athway associated **(MOCAP,** cluster M5;
486 cluster P2-P3, P7) cells: MOCAP cells (N=5) were characterized by somata located
487 exclusively in the iML, with aspiny or sparsely spiny bipolar dendritic trees that covered all
488 layers of the DG. The distinctive characteristic of MOCAP cells was that their axon projected
489 primarily to the iML (61.4 ± 16.9% of axon), co-aligned with the commissural-associational
490 pathway similar to HICAP cells. Unlike other ML IN classes, MOCAP cells displayed a
491 moderately hyperpolarized V_m (-63.2 ± 9.0 mV), a high R_{in} (287.4 ± 193.6 MΩ) and a long
492 membrane time constant (20.0 ± 14.8 ms). The AP discharge pattern revealed an adaptation
493 (ISI 1st/last: 0.73 ± 0.29) with slow AP kinetics (half-height duration: 1.03 ± 0.28 ms).

494 **HIPP L**ike (**HIPP L,** cluster M9; cluster P3, P7-P8) cells: These cells (N=4) showed similarity
495 to HIPP cells with respect to the somato-dendritic and axonal distributions, with horizontally
496 oriented somata in the hilus, spiny dendrites exclusively restricted to the same layer
497 (90.9 ± 12.4% of dendrites) and few dendrites reaching the GCL (4.5 ± 3.8% of dendrites).

498 HIPP L cells projected mainly to the outer two thirds of the ML (66.7 ± 25.5% of axon) with
499 the highest proportion observed in the mML (53.3 ± 26.4% of axon), but unlike true HIPP
500 cells also formed a substantial axon ramification in the hilus (19.6 ± 15.3% of axon). HIPP L
501 cells generally displayed a more depolarized V_m (-55.3 ± 8.0 mV) and a large voltage sag in
502 response to hyperpolarizing current pulses (13.6 ± 7.8% of max. voltage decrease at -250
503 pA). The AP discharge of HIPP L cells showed minimal adaptation (ISI 1st/last: 0.85 ± 0.21),
504 with small amplitude APs (59.4 ± 13.8 mV) and large fast AHPs (-24.1 ± 4.0 mV).

505 **H**ilar **P**rojecting (**HP**, cluster M10; cluster P1, P5 and P8) cells: HP cells (N=5) had somata
506 located in the hilus with sparsely spiny, radial dendrites extending to the ML. The axon of HP
507 cells was mainly restricted to the hilus (76.6 ± 20.4% of axon) with few collaterals crossing
508 the GCL (10.2 ± 13.0% of axon). The V_m of HP cells was relatively depolarized (-58.7 ± 6.4
509 mV) and they produced small amplitude APs (54.8 ± 9.9 mV) with moderate kinetics (half-
510 height duration: 0.72 ± 0.17 ms).

511 **Hi**lus **M**edial **P**erforant **P**athway associated (**HIMPP,** cluster M9-M10, M12; cluster P5, P7-
512 P8) cells: The HIMPP cell type (N=10) was characterized by somata located in the hilus with
513 aspiny or sparsely spiny dendrites covering all layers of the DG (% of dendrites: Hilus:
514 52.1 ± 13.8; GCL: 13.7 ± 5.9; ML: 34.2 ± 14.0). Neurons of this class gave rise to an axon
515 that preferentially innervated the mML (44.4 ± 20.0% of axon). Physiologically, HIMPP cells
516 had a relatively depolarized V_m (-59.2 ± 5.7 mV), high R_{in} (384.0 ± 119.5 MΩ) and a long
517 membrane time constant (29.8 ± 9.5 ms). Their AP discharge pattern showed slight
518 adaptation (ISI 1st/last: 0.78 ± 0.40), with intermediate AP kinetics (half-height duration:
519 0.73 ± 0.14 ms) and a large medium AHP (-12.8 ± 7.3 mV).

520 Finally, DGC (N=4) and MC (N=3) clusters clearly segregated from INs, reflecting the
521 divergent morphology and physiological properties. Indeed, in terms of physiology, DGCs
522 and MCs displayed a hyperpolarized V_m and slower membrane time constants than the
523 majority of DG INs. Furthermore, they displayed high AP voltage thresholds and in response
524 to depolarization, elicited large amplitude APs with a high degree of adaptation.

525 An overview of all morpho-physiologically identified neuronal clusters is presented in Figure 6
526 (see also Supporting Information Figures 10-21). Comparison of the morpho-physiological
527 with the morphology-alone dendrogram identified some notable differences in cluster
528 composition arising after the inclusion of physiological parameters. Such disparities were
529 particularly apparent in the SP I, HICAP and HIMPP classes. Large discrepancy to the
530 combined morpho-physiological cluster result, however, was observed in the physiology-
531 alone dendrogram with only P1, P3 and P6 revealing a high convergence to the identified IN
532 classes (Supporting Information Figure 2). A detailed summary of all morphological and

533 physiological characteristics of the identified morpho-physiological clusters can be found in
534 Tables 2-4 and in Supporting Information Tables 7-8 and Supporting Information Figure 9.

535 **3.6. Neurochemical marker expression in DG INs**

536 To confirm the IN types we report possessed distinctive neurochemical marker expression,
537 we performed immunohistochemical labeling of selected neurons (N= 64 neurons). The
538 expression of neurochemicals was homogenous in some classes, whilst others displayed
539 greater heterogeneity (Fig. 7). Specifically, the BC cluster showed uniform expression of PV
540 (5/5 cells tested). Similarly, the AAC cluster was predominantly PV expressing (4/5 cells
541 tested), but also contained a single (p)CCK expressing IN (1/5 cells tested). The SP II and
542 MOPP clusters expressed neuronal nitric oxide synthase (nNOS, SP II: 4/4 cells tested;
543 MOPP: 8/8 cells tested), while HIPP and HIPP L clusters displayed expression of SST
544 (HIPP: 3/3 cells tested; HIPP L: 4/4 cells tested). HICAP cells contained (p)CCK (1/2 cells
545 tested) and Calbindin (CB, 1/2 cells tested) which were also present among TML neurons
546 (CB: 4/9 cells tested; (p)CCK: 3/9 cells tested) besides an additional co-expression of CB
547 and neuropeptide Y (NPY) in this cluster (2/9 cells tested). While SP I and NGFC types
548 uniformly expressed nNOS (SP I: 5/5 cells tested; NGFC: 2/2 cells tested), other
549 neurochemicals were also co-expressed (SP I: NPY: 1/5 cells tested, Calretinin (CR): 1/5
550 cells tested; NGFC: NPY: 1/2 cells tested, CR: 1/2 cells tested). HP neurons displayed a
551 dichotomous marker expression with 2/5 cells immunoreactive for PV and 3/5 cells for
552 (p)CCK. MOCAP neurons were often immunoreactive for either (p)CCK (2/4 cells tested) or
553 nNOS (2/4 cells tested). Finally, HIMPP neurons were the most neurochemically
554 heterogeneous type and variably expressed nNOS (2/8 cells tested), (p)CCK (4/8 cells
555 tested) and CB (2/8 cells tested) with co-expression of NPY (1/8 cells tested)

556 Comparison with the neurochemical marker distribution displayed by the morphological and
557 physiological cluster results separately (Supporting Information Figure 3) revealed a similar
558 molecular pattern of the morphological result whereas much higher neuropeptide diversity
559 was observed among the physiological clusters.

560 **4. Discussion**

561 In this study, we show that DG INs are morphologically and physiologically diverse and can
562 be classified into distinct types. However, analysis based on morphology or physiology alone
563 is insufficient to describe the true diversity of DG INs. We reveal a more complete estimation
564 of this diversity by combining these two parameter sets which reliably distinguished
565 previously described IN types and demonstrated the existence of novel DG IN types.

566

567 **4.1. Morphology or physiology alone are insufficient to define DG IN diversity**

568 A central assumption regarding neuronal diversity is that form follows function. As such, most
569 studies examining IN diversity have defined and classified types based on their postsynaptic
570 target specificity, represented by axon localization at the light microscopic level (Booker and
571 Vida 2018; Buhl et al. 1994; DeFelipe et al. 2013; Freund and Buzsáki 1996; Han et al. 1993;
572 Pelkey et al. 2017) and thus ultimately on their control of the local neuronal network and
573 information transfer. With this in mind, we first sought to determine whether a purist
574 anatomical approach alone was sufficient to describe DG IN diversity. Based on a
575 reductionist morphological parameter space (20 axonal and 18 somato-dendritic
576 parameters), our analysis revealed 12 IN clusters of which 7 closely resembled previously
577 described types (Armstrong et al. 2011; Freund and Buzsáki 1996; Han et al. 1993; Hosp et
578 al. 2014; Mott et al. 1997). However, some clusters showed heterogeneity and included
579 multiple putative cell types.

580 Many studies, to date, have relied upon physiological properties of INs to confer identity, for
581 example fast-spiking vs. regular-spiking IN classes (Druckmann et al. 2013). Using a
582 reductionist classification method, as performed for morphology, we selected 15 passive and
583 active electrophysiological parameters of neurons that represented the vast majority of IN
584 diversity. However, subsequent clustering of INs based on these parameters only revealed 8
585 physiological types, inconsistent with the result of the morphological approach. This finding is
586 in good accord with previous studies that revealed physiology alone as a poor classifier of IN
587 diversity (Gouwens et al. 2020; Hosp et al. 2014). Nevertheless, morphological and
588 physiological classification of types did converge for at least certain IN types (M2-4 with P3,
589 M7 with P1). The remaining physiological types displayed a high degree of morphological
590 diversity, in agreement with Mott et al. 1997, who showed that physiological parameters do
591 not correlate well with axon distribution. That being said, physiological parameters were
592 sufficient to distinguish hilus and ML INs, with V_m typically more hyperpolarized in latter types.

593 **4.2. Morpho-physiological clustering better reflects the diversity of DG INs**

594 Our analysis of either morphology or physiology alone did not fully reflect the diversity of DG
595 INs and could not fully separate known types. Recent studies have shown that a combined
596 morpho-electrophysiological approach can better explain diversity (Gouwens et al. 2019;
597 Hosp et al. 2014). As such, we employed a morpho-physiological clustering approach, based
598 on the 53 morphological and electrophysiological parameters. This combined approach
599 increased the number of identified IN clusters to 13, including previously undescribed IN
600 types (Fig. 5 and 6). Enhanced classification was noted in a number of morphotypes when
601 physiology data was included, particularly in the M10 (INs with a major hilar projection) and
602 M12 cluster what could not be dissected by morphology alone. Indeed, our combined cluster
603 analysis identified most, if not all, previously described DG IN types (Han et al. 1993; Hosp et

604 al. 2014; Mott et al. 1997), including BCs, AACs, HIPP cells, HICAP cells, TML cells, NGFCs
605 and MOPP cells (with scattered atypical representatives observed among the latter type, i.e.
606 distinct hilar projection, see Supporting Information Figure 14). However, comparing our
607 morpho-physiological Ward´s minimum variance method cluster result with a different
608 clustering approach (k-means) revealed a high degree of correlation, but with merging of
609 HICAP and TML types. Although characterized by distinct axonal distributions, these results
610 could point out a larger morpho-physiological resemblance between these two IN classes
611 than previously reported (Yu et al. 2015) which could be also indicated by their shared
612 expression of (p)CCK and CB, observed in this study.

613 Furthermore, a number of novel IN types were identified, which included:

614 (1) SP I/II cells which possessed an axon predominantly located in the oML and also the
615 subiculum, which likely correspond to cells described by Ceranik et al. 1997. However, this
616 neuron class seems to comprise 2 distinct types. Besides major subiculum projections,
617 observed in both types, SP I neurons had a more vertically oriented axon also occasionally
618 extending into the hilus whereas SP II neurons revealed a more horizontal oriented axonal
619 distribution restricted to the mML and oML. Differences were also apparent in their
620 neuropeptide expression with SP I cells co-expressing NPY and CR together with nNOS in a
621 subset of cells, compared to an exclusive nNOS expression in SP II neurons, indicating their
622 potential molecular heterogeneity. Albeit not tested in the present study, these clusters may
623 also comprise INs expressing vasoactive intestinal peptide (VIP), which have recently been
624 shown as a diverse group in the DG, including a type with axon and dendrites extending into
625 the subiculum (Wei et al. 2021).

626 (2) MOCAP cells possessed HICAP like axon lamination, but with somata localized to the ML
627 and expressed a combination of (p)CCK and nNOS.

628 (3) HIMPP neurons had a layer specific axonal distribution similar to that of HIPP cells albeit
629 with an axon that was mainly restricted to the mML, co-aligned with the input from the medial
630 EC (Dolorfo and Amaral 1998; van Groen et al. 2003). Despite this specific axon alignment,
631 HIMPP cells possessed diverse neuropeptide markers, potentially indicating diverse
632 embryonic origins (Kepecs and Fishell 2014; Tricoire et al. 2011).

633 (4) HIPP L cells, like the related HIPP cell type, had an axon restricted to the outer two thirds
634 of the ML, most extensive within the mML and expressed SST, but, unlike HIPP cells, they
635 also densely ramified in the hilus. This cell type is likely representing a variant of HIPP cells,
636 but differs from the other SST neurons, HIL cells, recently described (Yuan et al. 2017),
637 which have local axons restricted to the hilus and form long-range projections to the septal
638 area.

639 (5) The cells, we identified as HP cells, with their somato-dendritic and axonal localization
640 confined to the hilus may reflect a closer approximation to HIL cells (Yuan et al. 2017).
641 However, the dichotomous expression of (p)CCK or PV in our sample indicates a
642 heterogeneity of these INs and may include BCs known to preferentially target MCs in the
643 hilus (Acsády et al. 2000). Indeed, MCs and a subset of hilar INs have been reported to form
644 a highly connected reciprocal network (Larimer and Strowbridge 2008). In contrast, HIL cells
645 have been shown to preferentially target other INs (Yuan et al. 2017) underscoring the
646 anatomical heterogeneity of hilar IN types. Nevertheless, given the partial co-expression of
647 PV and SST in INs from other hippocampal regions (Nassar et al. 2015; Booker et al. 2018),
648 HIL cells may be present among our PV expressing sample of HP cells.

649 Thus, by using a combined morpho-physiological clustering approach, we could identify
650 previously defined IN classes and several new IN types, in an objective, unbiased manner.
651 Intriguingly, the novel IN types displayed an axonal projection that was aligned with at least 1
652 layer of the DG (Fig. 8) emphasizing their functional implications in the hippocampal
653 microcircuit.

654 Our study sought to provide a detailed characterization of DG INs by combining a large
655 morpho-physiological parameter set with a cluster analysis. Such an extensive parameter set
656 potentially risks overfitting a given model, therefore we applied a KPCA to overcome the
657 confounds of multidimensionality by reducing the input variable dimensions. The relatively
658 low KMO values we report, together with the initial large parameter set may bias the outcome
659 of our cluster analyses with regard to the revealed cluster structures. Nevertheless, the fact
660 that we could clearly segregate PrCs from INs, not to mention previously identified IN
661 classes, supports the validity of our proposed model.

662 Besides the analytical limitation, our estimate of IN diversity is almost certainly an
663 underestimate, as we could not identify long-range projection INs (Eyre and Bartos 2019;
664 Melzer et al. 2012; Yuan et al. 2017), which possess axons cut during brain slicing.
665 Furthermore, several previously identified IN types were not detected in our study, for
666 example CCK BCs (Hájos et al. 1996), nor did we assess a full range of neurochemical
667 markers, as such may have overlooked important classifications, such as the various types
668 of VIP/CR INs (Acsády et al. 1996; Hájos et al. 1996; Wei et al. 2021). Additionally, whilst
669 this study provides detailed morpho-physiological characterization of 80 INs, greater
670 sampling may elucidate further functional diversity. Indeed, combining whole-cell recordings
671 and RNA-sequencing (Cadwell et al. 2017) with assessment of morphological, physiological
672 and transcriptomic features of neurons (Gouwens et al. 2020; Que et al. 2021; Scala et al.
673 2020) may allow greater determination of diversity.

674 **4.3. Functional ramifications of increased DG IN diversity**

The DG serves as a principal gateway to the hippocampal formation, transforming the high frequency, dense neural code from the EC into sparsified, orthogonal information for the downstream Cornu Ammonis (Hainmueller and Bartos 2020). This information transfer relies on the formation of stable ensembles of local DGCs, MCs and INs to process this incoming synaptic information (Hainmueller and Bartos 2018) in an afferent specific manner with respect to lateral vs. medial EC – which separately encode spatial and egocentric or non-spatial information, respectively (Hunsaker et al. 2007; Sewards and Sewards 2003; Witter et al. 2000). Previous studies have shown that multiple IN types have their inputs and outputs aligned with such specific paths, such as HICAP cells whose axon co-terminates with associative inputs in the iML or HIPP cells innervating the oML, which receives lateral EC inputs. Important to such afferent specific alignment is where IN dendritic fields align, such that HICAP and HIPP cells may be considered predominantly feed-back INs. The newly described HIPP L, HIMPP and MOCAP cells add to this diversity and layer specific control. HIPP L and HIMPP cells specifically co-terminate with medial EC inputs and thus may specifically control the spatial code in a feed-forward and feed-back manner given their diverse dendritic distribution. In contrast, MOCAP cells co-terminate with associative inputs, but likely produce such inhibition in a feed-forward manner.

Furthermore, the greater diversity of INs we described has implications for the activation of GABAergic receptors by a given IN type. Specifically, in DGCs slow $GABA_B$-receptors are preferentially localized to the distal dendrites in the mML and oML (Degro et al. 2015), contributing to activity dependent and tonic inhibition of these PrCs (Gonzalez et al. 2018). The layer specific and dense localization of the axon from these newly described and previously known DG INs will have direct ramifications on the spatio-temporal dynamics of GABAergic signaling achieved in both mature and immature DGCs (Markwardt et al. 2009). This further highlights the role of multiple IN classes for synaptic plasticity at a circuit level (Sambandan et al. 2010) and the profile of inhibition between PrCs and INs within the DG circuit more generally (Bartos et al. 2011).

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Conflict of Interest Statement:

The authors state that they have no competing financial interests.

Data availability statement:

711 The data that support the findings of this study are available from the corresponding author
712 upon reasonable request.

713

714 **Figure Legends:**

715 **Figure 1: VGAT-YFP expression and cell types of the dentate gyrus. A**. Overview of the
716 hippocampal formation (x4 and x20 magnification) taken from 300 µm thick transverse
717 hippocampal slices of vGAT-YFP rats. YFP-positive neurons can be found in all layers with
718 an abundance at the hilus-GCL border. YFP-negative neurons are found densely packed in
719 the GCL and scattered over the hilus. **B-D**. Reconstructions of YFP-positive neurons: a BC
720 (**B**), a HIPP cell (**C**), and an unknown cell type (**D**). **E-F**. Reconstructions of YFP-negative
721 neurons: a DGC in the GCL (**E**) and a MC from the hilus (**F**). **B-F**. Soma and dendrites are
722 shown in black and the axon in red. Insets illustrate voltage responses to a set of hyper- to
723 depolarizing current pulses (-250 pA to 500 pA, 50 pA steps, 500 ms duration) *(left)* and an
724 enlarged detail of the action potential discharge at 500 pA *(middle)*. Insets (*right*),
725 immunopositivity for PV (**B**), SST (**C**) and pCCK (**D**) in the biocytin filled cells. *Abbreviations:*
726 *vGAT, vesicular GABA transporter. YFP, yellow fluorescent protein. GCL, granule cell layer.*
727 *BC, basket cell. HIPP, hilar perforant pathway associated cell. DGC, dentate granule cell.*
728 *MC, mossy cell. PV, parvalbumin. SST, somatostatin. pCCK, pro-cholecystokinin. Bio,*
729 *biocytin. DG, dentate gyrus. iML, inner molecular layer. mML, middle molecular layer. oML,*
730 *outer molecular layer. CA, cornu ammonis.*

731 **Figure 2: Hierarchical cluster analysis of dentate gyrus interneurons based on their**
732 **morphological characteristics. A**. Eigenvalue/cumulative variance plot of the extracted
733 principal components. Principal components that were retained for clustering (N=7, *scree-*
734 *test, k-1*) are illustrated as red circles together with the kink-point of the graph (*k, red vertical*
735 *line*) and the contributed cumulative variance (*blue horizontal line*). **B**. Dendrogram of the
736 morphological cluster analysis using Ward's minimum variance method. The 14 different
737 clusters identified are illustrated by different colors (M1-M12, MC, DGC). M1: N=5, M2: N=8,
738 M3: N=5, M4: N=9, M5: N=6, M6: N=4, M7: N=5, MC: N=3, DGC: N=4, M8: N=3, M9: N=5,
739 M10: N=9, M11: N=8, M12: N=13. Inset represents the inverse scree plot (*no. of*
740 *clusters/merging level*) to define the optimal number of clusters that were maintained (*pale*
741 *red* area) based on the inflection point of the graph (*elbow-criterion*). **C**. Summary bar charts
742 of the layer-specific axonal (*red*) and dendritic (*grey*) distribution (as proportion of the total
743 length, %, mean ± SD) of each cluster aligned to the inset scheme (*left*). Soma localization is
744 indicated as a white diamond on the y-axis. The molecular layer (ML) is subdivided into
745 inner, middle and outer ML (*grey dashed lines*). *Abbreviations: PC, principal component. MC,*
746 *mossy cell. DGC, dentate granule cell. Sub, subiculum. GCL, granule cell layer.*

747 **Figure 3: Hierarchical cluster analysis of dentate gyrus interneurons based on their**
748 **physiological characteristics. A**. Eigenvalue/cumulative variance plot of the extracted
749 principal components. Principal components that were retained for clustering (N=5, *scree-*
750 *test, k-1*) are illustrated as red circles together with the kink-point of the graph (*k, red vertical*
751 *line*) and the contributed cumulative variance (*blue horizontal line*) **B**. Dendrogram of the
752 physiological cluster analysis obtained using Ward's minimum variance method. The 8
753 different clusters identified are illustrated by different colors (P1-P8). P1: N=11, P2: N=9, P3:
754 N=30, P4: N=4, P5: N=13, P6: N=3, P7: N=5, P8: N=12. Inset represents the inverse scree
755 plot (*no. of clusters/merging level*) to define the optimal number of clusters that were
756 maintained (*pale red* area) based on the inflection point of the graph (*elbow-criterion*). **C**.
757 Heatmap of the normalized physiological parameters plotted for each neuron (columns).
758 Physiological parameters are ordered based on an independent Ward clustering.
759 *Abbreviations: PC, principal component. AP, action potential. mAHP, medium after-*
760 *hyperpolarization. ISI, interspike interval. fAHP, fast after-hyperpolarization.*

761 **Figure 4: Electrophysiological properties of identified P-clusters and comparison with**
762 **the morphological cluster result. A.** Voltage responses to a set of hyper- to depolarizing
763 current pulses (-250 to 500 pA, 50 pA steps, 500 ms duration) with a representative single
764 action potential (detail) elicited at rheobase. **B.** I/V-plot of the different P-types (mean ± SD*)*
765 revealed differences among clusters ($p<0.0001$, 2-way-ANOVA, P1: N=11, P2: N=9, P3:
766 N=30, P4: N=4, P5: N=13, P6: N=3, P7: N=5, P8: N=12). **C.** Current-firing response of the
767 different P-types (mean ± SD) to a set of depolarizing current pulses revealed differences
768 among clusters ($p<0.0001$, 2-way-ANOVA, P1: N=8, P2: N=8, P3: N=30, P4: N=3, P5: N=11,
769 P6: N=3, P7: N=5, P8: N=11). **D-E.** Representative AP voltage response (**D**, *aligned*
770 *thresholds*) and phase plot (**E**, large panel) of a single neuron per P-cluster. Small panels in
771 (**E**) show phase plots of each neuron within the identified P-clusters*.* **F.** Convergence of the
772 morphological and physiological clusters of dentate gyrus neurons. Dendrogram illustrates
773 the morphological cluster result. Lower bars (color codes) represent the allocation of the
774 individual physiologically identified (P-types) neurons to the morphologically identified
775 clusters. *Abbreviations: MC, mossy cell. DGC, dentate granule cell.*

776 **Figure 5: Interneuron classes of the dentate gyrus based on a combined morpho-**
777 **physiological hierarchical cluster analysis. A.** Eigenvalue/cumulative variance plot of the
778 extracted principal components. Principal components that were retained for clustering (N=9,
779 *scree-test, k-1*) are illustrated as red circles together with the kink-point of the graph (*k, red*
780 *vertical line*) and the contributed cumulative variance (*blue horizontal line*) **B.** Dendrogram of
781 the combined morpho-physiological cluster analysis obtained using Ward's minimum
782 variance method. The 15 different clusters identified are illustrated by different colors. SP I:
783 N=7, NGFC: N=3, SP II: N=6, MOCAP: N=5, MOPP: N=11, BC: N=5, AAC: N=5, MC: N=3,

784 DGC: N=4, TML: N=9, HICAP: N=7, HP: N=5, HIMPP: N=10, HIPP L: N=4, HIPP: N=3. Inset
785 represents the inverse scree plot (*no. of clusters/merging level*) to define the optimal number
786 of clusters that were maintained (*pale red* area) based on the inflection point of the graph
787 (*elbow-criterion*). **C**. Heatmap of the normalized morpho-physiological parameters plotted for
788 each neuron (columns). Morpho-physiological parameters are ordered based on an
789 independent Ward clustering. *Abbreviations: PC, principal component. SP I, subiculum*
790 *projecting cell I. NGFC, neurogliaform cell. SP II, subiculum projecting cell II. MOCAP,*
791 *molecular layer commissural-associational pathway associated cell. MOPP, molecular layer*
792 *perforant pathway associated cell. BC, basket cell. AAC, axo-axonic cell. MC, mossy cell.*
793 *DGC, dentate granule cell. TML, total molecular layer cell. HICAP, hilar commissural-*
794 *associational pathway associated cell. HP, hilar projecting cell. HIMPP, hilar medial perforant*
795 *pathway associated cell. HIPP/ HIPP L, hilar perforant pathway associated (like) cell. CA3,*
796 *cornu ammonis 3. ISI, interspike interval. Sub, subiculum. oML, outer molecular layer. mML,*
797 *middle molecular layer. iML, inner molecular layer. GCL, granule cell layer. AP, action*
798 *potential. mAHP, medium after-hyperpolarization. fAHP, fast after-hyperpolarization.*

799 **Figure 6: Illustration of the morpho-physiologically identified interneuron clusters in**
800 **the dentate gyrus.** Representative reconstructions and voltage responses to a set of hyper-
801 to depolarizing current pulses (50 pA steps, 500 ms duration) of each identified cluster.
802 Soma and dendrites are shown in black, the axon in red. *Insets*, immunopositivity of the
803 different IN clusters is shown together with the biocytin filled soma. White scale bar: 20 µm.
804 *Abbreviations: SP I, subiculum projecting cell I. NGFC, neurogliaform cell. SP II, subiculum*
805 *projecting cell II. MOCAP, molecular layer commissural-associational pathway associated*
806 *cell. MOPP, molecular layer perforant pathway associated cell. BC, basket cell. AAC, axo-*
807 *axonic cell. MC, mossy cell. DGC, dentate granule cell. TML, total molecular layer cell.*
808 *HICAP, hilar commissural-associational pathway associated cell. HP, hilar projecting cell.*
809 *HIMPP, hilar medial perforant pathway associated cell. HIPP/ HIPP L, hilar perforant*
810 *pathway associated (like) cell. Bio, biocytin. nNOS, neuronal nitric oxide synthase. (p)CCK,*
811 *(pro)-cholecystokinin. PV, parvalbumin. CB, calbindin. SST, somatostatin.*

812 **Figure 7: Correlation of the neurochemical marker expression with the morpho-**
813 **physiologically identified interneuron clusters.** Dendrogram represents the morpho-
814 physiological cluster result using Ward's minimum variance method. Each immunopositive
815 neuron (N=64) is represented by a colored bar in the row of the tested molecular marker
816 (color-code). *Abbreviations: PV, parvalbumin (red). SST, somatostatin (dark blue). (p)CCK,*
817 *(pro)cholecystokinin (green). CB, calbindin (purple). CR, calretinin (yellow). NPY,*
818 *neuropeptide Y (gray). nNOS, neuronal nitric oxide synthase (light blue). SP I, subiculum*
819 *projecting cell I. NGFC, neurogliaform cell. SP II, subiculum projecting cell II. MOCAP,*
820 *molecular layer commissural-associational pathway associated cell. MOPP, molecular layer*

perforant pathway associated cell. BC, basket cell. AAC, axo-axonic cell. MC, mossy cell. DGC, dentate granule cell. TML, total molecular layer cell. HICAP, hilar commissural-associational pathway associated cell. HP, hilar projecting cell. HIMPP, hilar medial perforant pathway associated cell. HIPP/ HIPP L, hilar perforant pathway associated (like) cell.

Figure 8: Synopsis of identified interneuron classes in the dentate gyrus. Schematic overview of dentate gyrus interneuron types superimposed on the layered structure of the dentate gyrus (*grey*) with afferent pathways indicated by black arrows. Novel interneuron types are marked by an asterix (*). Somato-dendritic distributions are illustrated by oval surfaces and thick lines, the axonal distribution is shown as thin lines and circles. *Abbreviations: CA1, cornu ammonis 1. oML, outer molecular layer. mML, middle molecular layer. iML, inner molecular layer. GCL, granule cell layer. EC, entorhinal cortex. SP I, subiculum projecting cell I. SP II, subiculum projecting cell II. NGFC, neurogliaform cell. MOPP, molecular layer perforant pathway associated cell. MOCAP, molecular layer commissural-associational pathway associated cell. AAC, axo-axonic cell. BC, basket cell. HIPP/ HIPP L, hilar perforant pathway associated (like) cell. TML, total molecular layer cell. HICAP, hilar commissural-associational pathway associated cell. HIMPP, hilar medial perforant pathway associated cell. HP, hilar projecting cell (adapted and modified from Booker and Vida 2018).*

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