Electrophysiological and Molecular Characterization of the Parasubiculum

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The parahippocampal region is thought to be critical for memory and spatial navigation. Within this region lies the parasubiculum, a small structure that exhibits strong theta modulation, contains functionally specialized cells, and projects to layer II of the medial entorhinal cortex (MEC). Thus, it is uniquely positioned to influence firing of spatially modulated cells in the MEC and play a key role in the internal representation of the external environment. However, the basic neuronal composition of the parasubiculum remains largely unknown, and its border with the MEC is often ambiguous. We combine electrophysiology and immunohistochemistry in adult mice (both sexes) to define first, the boundaries of the parasubiculum, and second, the major cell types found in this region. We find distinct differences in the colabeling of molecular markers between the parasubiculum and the MEC, allowing us to clearly separate the two structures. Moreover, we find distinct distribution patterns of different molecular markers within the parasubiculum, across both superficial-deep and DV axes. Using unsupervised cluster analysis, we find that neurons in the parasubiculum can be broadly separated into three clusters based on their electrophysiological properties, and that each cluster corresponds to a different molecular marker. We demonstrate that, while the parasubiculum aligns structurally to some to general cortical principals, it also shows divergent features in particular in contrast to the MEC. This work will form an important basis for future studies working to disentangle the circuitry underlying memory and spatial navigation functions of the parasubiculum.

Key words: cell classification; electrophysiology; immunohistochemistry; parahippocampal network; parasubiculum

Significance Statement

We identify the major neuron types in the parasubiculum using immunohistochemistry and electrophysiology, and determine their distribution throughout the parasubiculum. We find that the neuronal composition of the parasubiculum differs considerably compared with the neighboring medial entorhinal cortex. Both regions are involved in spatial navigation. Thus, our findings are of importance for unraveling the underlying circuitry of this process and for determining the role of the parasubiculum within this network.

Introduction

The parasubiculum (PaS) is a small, elongated structure within the parahippocampal region, lying between the presubiculum (PrS) and the medial entorhinal cortex (MEC). The parahippocampal region is thought to play an integral role in spatial

navigation and has attracted attention since the discovery of grid cells (Hafting et al., 2005). While the majority of work studying spatial navigation has focused on the hippocampus and MEC, the discovery of functional cell types in both the PrS and PaS has led to an increasing interest in these structures too. The PaS contains grid cells, head-direction cells, and border cells (Taube, 1995; Boccara et al., 2010), but it is the position of the PaS in the circuitry that perhaps makes it most interesting for the study of spatial navigation. The PaS receives inputs from the anterior thalamus (van Groen and Wyss, 1990; Ding, 2013), where an abundance of head-direction cells is found (Taube, 2007; Winter et al.,

Received April 9, 2019; revised Aug. 30, 2019; accepted Sept. 4, 2019.

Author contributions: R.P.S., D.P., and D.S. designed research; R.P.S., D.P., and A.S. performed research; R.P.S. and D.P. analyzed data; R.P.S. wrote the first draft of the paper; R.P.S., D.P., A.S., and D.S. edited the paper; R.P.S. wrote the paper.

This work was supported by Deutsche Forschungsgemeinschaft Grants SFB 958 and Exc 257, Bundesministerium für Bildung und Forschung Bernstein Center for Computational Neuroscience Berlin Grant 01GQ1001A, and Bernstein Focus Learning Grant 01GQ0972. We thank Susanne Rieckmann and Anke Schönherr for excellent technical assistance; Dr. Atsushi Miyawaki for providing the pCS2-Venus plasmid; Dr. Yuchio Yanagawa for generous donation of VGAT-Venus mice; Prof. Susumu Tonegawa for generous donation of WFS1-Cre mice; and Jörg Breustedt, Constance Holman, and John Tukker for discussions and comments on the manuscript.

The authors declare no competing financial interests.

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<https://doi.org/10.1523/JNEUROSCI.0796-19.2019>

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Table 1. Transgenic mouse lines used for acute electrophysiology experiments^a

Line	No. of slices (animals)	RRID/source	Reference
CS7-BL6nCrI	90 (42)	IMSR_CRL:027	
VGAT-Venus	30 (15)	Yuchio Yanagawa lab	Wang et al., 2009
VGAT-Cre	8 (4)	IMSR_JAX:017535	Chao et al., 2010
x Ai9		IMSR_JAX:007909	Madisen et al., 2010
WFS1-Cre	2 (2)	Susumu Tonegawa lab	Kitamura et al., 2014
ChaT-Cre	88 (25)	MMRRC_037336-UCD	Gong et al., 2007
PV-Cre	43 (10)	IMSR_JAX:008069	Hippenmeyer et al., 2005

^aSome data are taken from recorded cells that were part of other studies and, thus, were not always from WT or VGAT-Venus animals.

2015). Additionally, PaS receives input from the medial septum (Tang et al., 2016), a major generator of theta oscillations, as well as the subiculum, and CA1 (van Groen and Wyss, 1990; Ding, 2013; O'Reilly et al., 2013). The PaS sends output projections to layer 2 (L2) of the MEC (Köhler, 1985; van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993), where the greatest abundance of grid cells are found (Sargolini et al., 2006), as well as to the lateral entorhinal cortex, ipsilateral and contralateral PrS, and the contralateral PaS (van Groen and Wyss, 1990; Agster and Burwell, 2013). Current research is addressing how grid cells develop their distinctive firing patterns and how this circuit combines information from functionally different cell classes to encode spatial information. Thus, given the upstream and downstream targets of the PaS, it is uniquely positioned to influence both the development and continuing modulation of grid cells in the MEC. Moreover, lesion studies have shown that damage to the PrS and PaS impairs spatial location memory (Kesner and Giles, 1998; Liu et al., 2004), highlighting the importance of the PaS in the navigation circuit.

To address exactly how the PaS is involved in navigation and memory processes, it is important that we first understand what the basic components of this brain region are. Despite our increasing knowledge of the functional cell types within the PaS, our understanding of the basic, cellular composition of this region remains sparse. In this study, we use a combination of immunohistochemistry and electrophysiology to identify the major cell types within the mouse PaS. The PaS has previously been reported to express high levels of the transmembrane protein Wolframin, encoded by the gene *WFS1* (Luuk et al., 2008; Ramsden et al., 2015; Ray et al., 2017). Here, we confirm these findings and show that *WFS1*-expressing cells comprise the principal, putative excitatory population of neurons and make up the majority of neurons in the PaS. In addition, we find that the PaS contains a heterogeneous population of interneurons, with different molecular subtypes showing different distributions along the superficial to deep axis. Electrophysiological data support these findings, with neurons falling into three clusters based on their passive and active properties. Two of these clusters represent putative inhibitory neurons, while the third, largest cluster represents the principal cell population of the PaS.

Materials and Methods

Experimental design and statistical analysis. To obtain both an anatomical and a physiological overview of the PaS, we performed immunohistochemistry and *in vitro* slice electrophysiology. For our immunohistochemistry experiments, we used adult vesicular GABA transporter-Venus (VGAT-Venus) transgenic mice (Wang et al., 2009) of both sexes (postnatal day 150 or older, $n = 4$ animals). For our electrophysiology experiments, we prepared slices from adult mice of both sexes (postnatal day 150 or older, $n = 98$ animals) from several different genetic mouse lines (Table 1) (Hippenmeyer et al., 2005; Gong et al., 2007; Wang et al., 2009; Chao et al., 2010; Madisen et al., 2010; Kitamura et al., 2014). Further details of

Table 2. Primary antibodies used in immunolabeling

Antibody	Species	Dilution	Supplier, catalog
Calbindin	Mouse monoclonal	1:5000	Swant, 300
Calbindin	Rabbit polyclonal	1:5000	Swant, CB38
Calretinin	Mouse monoclonal	1:2000	Millipore, MAB1568
CCK	Rabbit polyclonal	1:1000	Sigma-Aldrich, C2581
GFP	Chicken polyclonal	1:1000	Abcam, ab13970
NPY	Rabbit polyclonal	1:1000	Immunostar, 22940
NeuN	Mouse monoclonal	1:1000	Millipore, MAB377
PV	Mouse monoclonal	1:1000	Swant, PV235
Reelin	Mouse monoclonal	1:1000	Millipore, MAB5364
SOM	Rabbit polyclonal	1:1000	Bachem, T4102
VIP	Rabbit polyclonal	1:1000	Immunostar, 20077
WFS1	Rabbit polyclonal	1:1000	Proteintech, 11558-1-AP

sample sizes for individual experiments are reported in the figure legends. All animal maintenance and experiments were performed in accordance with institutional guidelines, guidelines of the local state government (Berlin state government, T0100/03; O0413/12), and the European Union Council Directive 2010/63/EU. The study was not pre-registered. Statistics were performed in Python using *Scipy* and *Statsmodels* packages. Data were first checked for normality using a Shapiro–Wilk test. Since data were not normally distributed, nonparametric Kruskal–Wallis tests were subsequently performed to test for differences in parameter values across groups, and *post hoc* Dunn's test performed to determine specific group differences. Significance levels were set to 0.05, for multiple-comparisons *p* values were adjusted using the false discovery rate (Benjamini/Hochberg) correction method (Benjamini and Hochberg, 1995). All values are reported as median, interquartile range (IQR) unless otherwise stated.

Immunohistochemistry. Immunohistochemistry was performed on horizontal sections from VGAT-Venus transgenic mice (Wang et al., 2009), to enable cell density counts and labeling of different inhibitory populations. Mice were initially anesthetized with isoflurane, followed by an intraperitoneal injection of ketamine/xylazine (100 mg/15 mg per kg) and transcardially perfused with 0.1 M PBS, pH 7.4, followed by 4% PFA. Brains were harvested and stored in 4% PFA overnight. The following day, brains were rinsed in PBS and sectioned using a vibrating microtome slicer (Leica Microsystems, VT 1200S). Sections of 100 μ m were cut and dropped into PBS after slicing. Free-floating sections were washed 3 times in PBS (5 min each) and incubated in a blocking solution composed of 5% NGS (Biozol), 1% Triton-X (Sigma-Aldrich), and PBS, for 3 h at room temperature with gentle agitation. Primary antibodies (Table 2) were diluted in blocking solution (2.5% NGS, 1% Triton-X, PBS), and sections were incubated for 72 h at 4°C. Following this, sections were washed 3 times (20 min each) with PBS and secondary antibodies (goat anti-rabbit AlexaFluor-647 and goat anti-mouse AlexaFluor-555, Invitrogen; 1:500 dilution in PBS) were applied for 3 h at room temperature. Finally, slices were washed 4 times (15 min) with PBS, before being mounted on glass slides in mounting medium (Mowiol). We found antibodies to have good tissue penetration in slices of 100 μ m thickness. Any slices where clear antibody signal could not be detected throughout at least a continuous 50 μ m stack in the slice were discarded and not used for cell counts.

Slices that had been prepared for electrophysiology and contained recorded and filled cells were fixed overnight in 4% PFA. Sections (400 μ m thickness) were then washed 3 times (5 min each) in PBS and stained using the same protocol. To label the biocytin, a conjugated streptavidin marker was used (conjugated to AlexaFluor-488 or AlexaFluor-647). Up to three different primary antibodies were used in addition to the biocytin labeling. Secondary antibodies used were goat anti-rabbit AlexaFluor-405, goat anti-rabbit AlexaFluor-647, goat anti-mouse AlexaFluor-555 or goat anti-guinea pig AlexaFluor-555, and goat anti-chicken AlexaFluor-488 (Invitrogen).

Confocal microscopy and cell counting. Mounted sections were imaged on an upright TCS SP5 confocal microscope (Leica Microsystems). The following laser lines were used to illuminate fluorescent samples through a 20 $\times$ immersion objective (0.7 NA; Leica Microsystems): 405 nm (di-

ode), 488 nm (Argon laser), 568 nm (solid state), and 633 nm (Helium, Neon). For cells that had been filled with biocytin, a further image of the cell soma was obtained through a 63 $\times$ objective (1.4 NA; Leica Microsystems) to identify any *post hoc* immunostaining. All images were acquired at 1024 $\times$ 1024 pixels (pixel size, 0.72 and 0.23 μ m for 20 $\times$ and 63 $\times$ objectives, respectively). For cell counting, 50 μ m stacks were taken with a 0.49 μ m step size. Analysis of images was performed in Fiji (<https://imagej.nih.gov/ij/>). To calculate cell densities, a 100 $\times$ 100 μ m box was placed over the PaS. The box was first placed as close to the pia as possible, at a position where the distance between the pia and alveus was judged to be the greatest. To avoid potential overlap with the medial-most MEC (mMEC), the box was preferentially placed on the more medial side of the PaS. Cells within this frame were counted according to optical dissector counting rules (West et al., 1991); exclusion lines were drawn on the bottom and left-hand edges of the box, and inclusion lines on the upper and right-hand edges. Cells that touched either exclusion line were excluded, whereas cells touching inclusion lines were included in counts. Only cells coming into focus in the image stack were counted. The grid was moved throughout the depth of the PaS, starting at the pial surface and moving 100 μ m away from the pia, toward the alveus, each time, until part of the grid was no longer in the PaS (i.e., superficial to deep axis). Cell densities were calculated by multiplying the cell count by the volume covered by the grid. Shrinkage of sections was not taken into account.

Model fitting. GLMs were fit in python using the *statsmodels* package (www.statsmodels.org). Models were generated with a negative binomial distribution and a log-link function. Several models were created, and the most appropriate was decided upon by comparison of the Akaike information criteria (Akaike, 1974) and maximum likelihood tests between nested models. Model validation was performed using the leave-one-out cross-validation method. Differences between cell counts at different depths and for WFS1 and VGAT-Venus neurons, or different interneuron markers were concluded by nonoverlapping 95% CIs for predicted cell counts, using data predicted by the final chosen GLM.

Morphological reconstructions. For morphological reconstructions, tiled image stacks of the biocytin-filled neurons were taken using a 63 $\times$ objective. Images were taken at 1024 $\times$ 1024 pixels (pixel size 0.23 μ m) and with a z step size of 0.49 μ m. Multiple image tiles were stitched together in Fiji using the grid stitching plugin (Preibisch et al., 2009). Following acquisition, images were loaded into NeuTube (Feng et al., 2015) and reconstructed semiautomatically using the software's tracing tools.

Whole-cell patch-clamp recording. For whole-cell patch-clamp recordings acute brain slices were prepared from adult (postnatal day 50 and over, both sexes) WT mice (C57BL/6n, $n = 42$) or adult transgenic mice (Table 2). Animals were anesthetized under isoflurane and decapitated. Brains were removed and transferred to an ice-cold, sucrose-based dissection ACSF containing the following (in mM): 87 NaCl, 26 NaHCO₃, 50 sucrose, 10 glucose, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, and 3 MgCl₂, and saturated with 95% O₂ and 5% CO₂. Horizontal slices 400 μ m thick were cut across the whole span of the PaS (~6 slices per hemisphere) using a vibrating microtome slicer (Leica Microsystems, VT1200S). Once cut, slices were transferred to an interface chamber, which was continuously perfused with ACSF containing the following (in mM): 119 NaCl, 26 NaHCO₃, 10 glucose, 2.5 KCl, 1.3 MgCl₂, 1 NaH₂PO₄, and 2.5 CaCl₂, saturated with 95% O₂ and 5% CO₂, and maintained at 32°C–34°C. Slices were allowed to recover for at least 1 h before being transferred to a recording chamber. Recordings were performed in a submerged chamber, kept at 32°C–34°C, and perfused with the same ACSF as used for interface storage. Somatic whole-cell recordings were performed using glass pipettes pulled from borosilicate glass capillaries with a tip resistance of 3–6 M Ω . Pipettes were pulled using a horizontal DMZ Universal Puller (Carl Zeiss). Pipettes were filled with an internal solution containing the following (in mM): 120 K-Gluconate, 10 HEPES, 10 KCl, 5 EGTA, 2 MgSO₄·7H₂O, 3 MgATP, 3 NaGTP, and 5 phosphocreatine Na. Internal solution also contained 0.2% biocytin, to allow for later recovery and identification of recorded neurons. To identify cells for recording, slices were visualized using infrared differential interference contrast microscopy through a digital camera (XM10-IR, Olympus). In some cases, Venus-expressing inhibitory neurons were targeted using epifluorescence illumination by a mercury arc lamp (Olympus). Recordings

were performed using Multiclamp 700A/B amplifiers (Molecular Devices). Signals were filtered at 10 kHz and sampled at 20 kHz and either digitized using Digidata 1550 and recorded in pClamp10 (Molecular Devices) or using a BNC-2090 interface board (PCI 6035E A/D Board, National Instruments) and recorded in IGOR Pro 6.12 (WaveMetrics). Series resistance was monitored throughout recordings, and cells were discarded if the series resistance exceeded 25 M Ω . Pipette capacitance neutralization and bridge balance were applied and adjusted as appropriate. Liquid junction potential was not corrected for. Upon obtaining a whole-cell patch, neurons were characterized, in current-clamp configuration, by injecting increasing steps of negative and positive current. Current injections were applied for 1 s, in increasing steps of 40 pA. The voltage responses of neurons to these current injections were used to calculate intrinsic properties and action potential (AP) parameters.

Electrophysiological analysis. Electrophysiological features were extracted from recordings using custom-written routines in IGOR Pro. Resting membrane potential (V_m) was taken as the mean value of the baseline before current injections were performed. Input resistance was taken as the slope of the current-voltage fit between all negative and subthreshold positive current steps. Rheobase was estimated by fitting a line through the relationship between injected current and AP number and extrapolating this backwards. AP threshold was defined as the membrane potential at the point where dV/dt reached 5% of the maximum dV/dt. AP latency was calculated as the time between current injection and the first AP onset. AP height was taken as the difference between AP threshold and maximal voltage of first AP. The rising and falling slopes of APs were taken as the maximal and minimal differential values, respectively, and these two values used to calculate the slope ratio. AP half-width was taken as the time between the half-height of the upward and downward phases. The interval between AP2 and AP3 was calculated as the time between onset of each of the second and third APs. The same was calculated for AP9 and AP10, using the first current injection step that elicited at least 10 APs. The adaptation index was then calculated by dividing the interval of AP9–AP10 by the interval of AP1–AP2. If a cell did not produce 10 APs with maximal current injection, then the maximum number of APs were used to calculate the last interval, instead of AP9–AP10. A relative firing frequency was calculated by taking the firing frequency two current steps above the rheobase value. Cells were considered as inactivated when current injection no longer produced APs that reversed >0 mV. Afterhyperpolarization (AHP) was defined as the difference between AP threshold and the minimum voltage seen immediately after the AP (within 2 ms). The medium AHP was taken as the difference between baseline V_m and the minimum voltage seen up to 200 ms after the end of current injection. A sag ratio was calculated using 1 s steps of negative current injection and dividing the voltage difference between baseline and the steady-state hyperpolarization by the maximal voltage deflection immediately after the current injection onset. Theta power was calculated by applying a fast Fourier transform to the trace of the highest current injection not producing any APs. For each cell, the maximum power and corresponding frequency in the theta range (3–12 Hz) was taken.

Cell classification and cluster analysis. Hierarchical clustering and principal component analysis were performed using the following electrophysiological parameters: V_m , input resistance, sag ratio, rheobase, input–output slope, AP latency, AP2–AP3 interval, AP9–AP10 interval, minimum interspike interval, AP threshold, AP amplitude, AP half-width, adaptation index, medium AHP, AHP, relative firing frequency, maximum depolarization rate, maximum repolarization rate, and the ratio of maximum and minimum dV/dt. Data were first standardized by centering (subtracting the mean of a parameter from each data point of the given parameter) and scaling (dividing by the SD). Clustering was based on Ward's method (Ward, 1963). Clustering was implemented using the *prcomp* function in the *stats* package in R (<http://www.r-project.org/>). Euclidean distance was calculated using the scaled and centered data and the distance matrix used to compute the hierarchical clustering. To estimate the optimal number of clusters, we used the gap statistic method, whereby the within-cluster dispersion was compared with that from a reference distribution over 500 trials, for $k = 1$ to 10 clusters. The reference distribution was randomly assigned within the same range as our dataset. The mean and SD of the gap between the

Figure 1. Delineation of the PaS. **A**, Horizontal section labeled with NeuN showing the parahippocampal region. Scale bar, 200 μm . **B**, WFS1 labeling of a horizontal slice, showing the high expression in the PaS and L2 of the MEC. Dotted lines outline the lateral and medial borders of the PaS, as well as L2 of the MEC and the fiber tract. WM, White matter. Scale bar, 200 μm . **C**, Closeup of WFS1 labeling in the PaS, showing the change in projection angle of apical dendrites of WFS1-expressing cells between the PaS and neighboring mMEC. (Figure legend continues.)

intracenter variation from our dataset, and that from the reference distribution was calculated across the 500 trials and the optimal number of clusters defined as the first peak where $\text{Gap}(k)$ was greater or equal to $\text{Gap}(k+1) - \text{SD}(k+1)$. The Calinski–Harabasz (CH) index was calculated to examine the ratio of intercluster and intracenter variability. CH index was calculated using the *calinski_harabasz_score* function from the *sklearn* package in Python. Cells were divided into clusters according to distance. Clusters containing <3 cells were merged with the most correlated cluster (Gouwens et al., 2019).

Results

Delineation of the PaS/MEC border using coexpression of markers

The PaS is situated between the PrS and the MEC in the parahippocampal region (Fig. 1A). As previously reported (Luuk et al., 2008; Ramsden et al., 2015; Ray et al., 2017), we found the protein marker WFS1 to be highly expressed in the PaS (Fig. 1B). On the medial side, a clear border can be distinguished between the PrS and PaS, with the PrS distinctly lacking WFS1 expression. On the lateral side, the distinction between the PaS and the MEC is less obvious, with pyramidal neurons in L2 of the MEC also expressing WFS1 and seemingly merging with the lateral edge of the PaS (Fig. 1B,C). However, we find several molecular distinctions between the two regions that may be used to delineate the PaS from the MEC. We made use of a transgenic mouse line, *VGAT-Venus* (Wang et al., 2009), to identify the inhibitory population of neurons. The most striking difference between the PaS and the MEC is the proportion of reelin cells that colocalize with *VGAT-Venus* across the two regions (Fig. 1D). In the PaS, we find 88% of reelin-labeled neurons overlap with *VGAT-Venus* expression. In contrast, in the MEC we find the inverse, with only 17% of reelin cells colocalizing with *VGAT-Venus* (Fig. 1E). A second clear distinction between the two regions can be seen in the colocalization of calbindin with WFS1 and *VGAT-Venus* (Fig. 1F). In the MEC, calbindin is often used alongside WFS1 as a marker for L2 pyramidal neurons (Ray et al., 2014; Kitamura et al., 2015; Sun et al., 2015). We checked for overlap between calbindin, WFS1, and *VGAT-Venus* expression in neurons in both the MEC and PaS (Fig. 1G,H). We find that, in the PaS, calbindin is exclusively inhibitory (100% of calbindin cells were *VGAT-Venus* positive; Fig. 1G). In the MEC, calbindin colocalizes with both WFS1 and *VGAT-Venus* in a mutually exclusive manner (calbindin-WFS1 overlap: 35%; calbindin-*VGAT-Venus* overlap: 37%; Fig. 1G). Meanwhile, in the PaS, there is no overlap between WFS1 and

calbindin staining, compared with in the MEC where 74% of WFS1 cells were colabeled with calbindin (Fig. 1H).

We also made two observations suggestive of a transitional zone between the PaS and MEC, which we believe corresponds to the previously reported mMEC (Fujimaru and Kosaka, 1996; Ray et al., 2017). While in the MEC WFS1 is expressed only in L2 neurons, at the interface between PaS and MEC, WFS1 can be seen across all superficial layers. Two properties appear to separate these WFS1 neurons from the WFS1 neurons seen in the PaS. First, in this region, there is colocalization of WFS1 and calbindin (Fig. 1Fii). Second, the orientation of the apical dendrite of WFS1 cells in this region appear orthogonal to those expressing WFS1 in the PaS (Fig. 1C). We observed this phenomenon in at least 19 slices where multiple cells had been filled with biocytin in the PaS and at the interface with the MEC. Combining these observations, we consider this region to be more alike to the MEC than the PaS; therefore, in the present study, we exclude this region from our analysis of the PaS.

Cellular composition of the PaS

We next looked at the neuronal composition of the PaS. To this end, horizontal sections containing PaS cut from the perfused brains of *VGAT-Venus* mice were immunostained with antibodies for NeuN and WFS1 (Fig. 2A). This enabled us to look at the total proportion of neurons expressing either WFS1 or *VGAT-Venus*. The PaS has been previously referred to as both a three-layered (Mulders et al., 1997; Burgalossi et al., 2011; Tang et al., 2016) and a six-layered structure (Funahashi and Stewart, 1997; Glasgow and Chapman, 2007; Boccara et al., 2010). Instead of assigning layers directly, we used a binning approach to look at cell densities at different distances from the pial surface to determine whether this gave indications of the laminar structure of the PaS. Neurons were counted at 100 μm increments from the pial surface (Fig. 2A), up to 500 μm from the pia. The total number of neurons (identified by NeuN label), as well as the number of WFS1 and *VGAT-Venus*-expressing neurons were counted (Fig. 2B). To allow for valid statistical comparisons across depth and cell type, we modeled our cell count data using a negative binomial GLM and found that both immunomarker (referred to in models as “stain”) and depth from the pia, as well as their interaction, were significant factors contributing to cell count. We tested several models and compared them using the Akaike Information Criteria (AIC) and deviance (Fig. 2C). Our data were best fit by a cubic expression that included staining, depth, and the interaction of these two terms, indicating that both molecular marker and location along the superficial-deep axis influence cell count ($\text{cell count} \sim \text{stain} \times \text{depth} + \text{stain} \times \text{depth}^2 + \text{depth}^3$, where * denotes the inclusion of each individual factor as well as their interactions; Table 3). We used the CIs of estimated cell density, predicted from our model, to compare WFS1 and *VGAT-Venus* distributions with each other and within themselves across the depth of the PaS (Fig. 2D). Using this approach, we found that the distribution of WFS1 labeling followed a nonuniform distribution throughout the superficial-deep axis of the PaS. We found WFS1 density to be at its lowest in the most superficial and deepest portions of the PaS, whereas the highest proportion of WFS1 was seen across the center of the PaS, ~ 100 – $400 \mu\text{m}$ from the pial surface (mean $\pm$ SEM WFS1 density at 0–100 μm : $5516 \pm 818 \text{ cells/mm}^3$; 100–200 μm : $16,368 \pm 689 \text{ cells/mm}^3$; 200–300 μm : $16,776 \pm 574 \text{ cells/mm}^3$; 300–400 μm : $12,400 \pm 737 \text{ cells/mm}^3$; 400–500 μm : $5188 \pm 515 \text{ cells/mm}^3$). The WFS1 density distribution followed closely the distribution of NeuN-labeled neurons, reflecting that the majority of the PaS neuronal population is made up of putatively excitatory, WFS1-expressing

←

(Figure legend continued.) Dashed arrows indicate the general projection direction of dendrites of PaS and mMEC neurons. Solid arrow indicates location of MEC. Inset, Biocytin labeling of two filled neurons: one in the PaS and one in the mMEC. Scale bars, 100 μm . **Di**, Stitched images of a horizontal slice labeled with reelin (magenta) and *VGAT-Venus* (green). Scale bars, 200 μm . **Dii**, Zoom-in of regions indicated by boxes in merged image of **Di** showing colocalization of reelin with *VGAT-Venus* in PaS and lack of colocalization in MEC. Scale bar, 20 μm . **E**, Percentage of reelin cells in PaS and MEC that colocalize with *VGAT-Venus*. Reelin-*VGAT-Venus* double-labeled cells as a percentage of all reelin cells: 88% in PaS; 17% in MEC. $N = 228$ cells, 15 slices, 4 animals (PaS) and 545 cells, 13 slices, 4 animals (MEC). **Fi**, Labeling of a slice with WFS1 (cyan), calbindin (magenta), and *VGAT-Venus* (green) in PaS and MEC. Scale bar, 200 μm . **Fii**, MEC, mMEC, and PaS zoomed regions correspond to colored boxes in **Fi**. Scale bar, 20 μm . **G**, Percentage of colocalization between calbindin and either *VGAT-Venus* or WFS1 in the PaS and MEC. Calbindin-*VGAT-Venus* double-labeled cells as a percentage of all calbindin cells: 100% in PaS; 37% in MEC. Calbindin-WFS1 double-labeled cells as a percentage of all calbindin cells: 0% in PaS; 35% in MEC. $N = 194$ cells, 8 slices, 2 animals (PaS) and 262 cells, 5 slices, 2 animals (MEC). **H**, Percentage of colocalization between WFS1 and calbindin in PaS and MEC. WFS1-calbindin double-labeled cells as a percentage of all WFS1 cells: 0% in PaS; 74% in MEC. $N = 1665$ cells, 8 slices, 2 animals (PaS) and 124 cells, 5 slices, 2 animals (MEC).

Figure 2. Cellular composition of the PaS. **A**, Example image of a single horizontal section labeled with WFS1 (left), NeuN (middle), and VGAT-Venus (right). Dotted lines indicate PaS borders and example placement of boxes used for cell counting. Scale bar, 100 μ m. **B**, Raw cell counts in 100 μ m bins from pia to deep PaS. Each circle represents count from one counting box. Lines indicate mean (horizontal) and SD (vertical). **C**, Comparison of AIC (gray) and deviance (black) for each of the six tested models. Models were created in an additive, semineested manner such that the models are composed of the following: M1: count $\sim$ stain*depth; M2: M1 + depth²; M3: M2 + depth³; M4: M2 + stain*depth²; M5: M4 + depth³; M6: M5 + stain*depth³, where * indicates the combined inclusion of both predictors as well as their interaction term. *p* values indicate outcome of likelihood ratio test performed between nested models. **D**, Mean and 95% CIs of estimated cell densities for NeuN (magenta), WFS1 (cyan), and VGAT (green) neurons, predicted using the selected model. **E**, Percentage of all NeuN-labeled neurons expressing WFS1 (cyan), VGAT-Venus (green), or neither (magenta), across varying depths of the PaS. 0–100 μ m: 62% WFS1, 31% VGAT-Venus,

neurons (Fig. 2E). In the central region of the PaS (200–300 μ m from pia), WFS1-positive cells made up 80% of all neurons. VGAT-Venus exhibited a different distribution, showing a relatively constant density across the middle body of the PaS, with the lowest density seen in the most superficial region and the highest density seen at the furthest away region from the pia (Fig. 2D). Despite having the lowest VGAT-Venus density in the most superficial region, inhibitory neurons accounted for 31% of all neurons in this sparsely populated area (Fig. 2E). In the deepest region (400–500 μ m from the pia), where VGAT-Venus was at its highest density, inhibitory neurons accounted for 38% of all neurons. Expression of WFS1 and VGAT-Venus was entirely nonoverlapping, suggesting that the WFS1 population, which makes up the majority of neurons in the PaS, is putatively excitatory.

We also looked to determine whether the DV axis had an influence on cell count, since it is known that other structures within the parahippocampal region show differences across this axis. Slices were compared with a reference map of horizontal slices, and, using landmark fea-

n = 635 cells, 12 slices, 4 animals; 100–200 μ m: 71% WFS1, 16% VGAT-Venus, *n* = 1452 cells, 12 slices, 4 animals; 200–300 μ m: 80% WFS1, 15% VGAT-Venus, *n* = 1075 cells, 9 slices, 4 animals; 300–400 μ m: 68% WFS1, 20% VGAT-Venus, *n* = 971 cells, 9 slices, 4 animals; 400–500 μ m: 39% WFS1, 38% VGAT-Venus, *n* = 504 cells, 7 slices, 4 animals. **F**, Comparison of AIC (gray) and deviance (black) scores for each of the 10 tested models, including DV slice position. Models are composed of the following: M1: count $\sim$ stain*depth*slice position; M2: M1 + depth²; M3: M2 + depth³; M4: M2 + depth²*stain; M5: M4 + depth³; M6: M5 + depth³*stain; M7: M4 + depth²*stain*slice position; M8: M5 + depth³*stain*slice position; M9: M7 + depth³; M10: M9 + depth³*slice position. *p* values indicate outcome of likelihood ratio test performed between nested models. **G**, Mean and 95% CIs of estimated cell densities for WFS1 at ventral (v), intermediate (i), and dorsal (d) levels of the PaS. *N*, Number of slices on which data are modeled: v = 8, i = 19, d = 10 for WFS1. **H**, Mean and 95% CIs of estimated cell densities for VGAT-Venus at dorsal, intermediate, and dorsal levels of the PaS. *N*, Number of slices on which data are modeled: v = 12, i = 30, d = 19 for VGAT-Venus. **I**, Cell count ratio of WFS1 to VGAT-Venus at ventral, intermediate, and dorsal levels of PaS. Mean $\pm$ SEM at 0–100 μ m: (v) 3.1 ± 0.4 , *n* = 653 cells, 9 slices, (i) 1.8 ± 0.4 , *n* = 504 cells, 16 slices, (d) 1.6 ± 0.3 , *n* = 202 cells, 9 slices; 100–200 μ m: (v) 3.6 ± 0.5 , *n* = 972 cells, 9 slices, (i) 5.2 ± 0.4 , *n* = 1653 cells, 16 slices, (d) 5.2 ± 0.5 , *n* = 772 cells, 9 slices; 200–300 μ m: (v) 3.7 ± 0.5 , *n* = 442 cells, 4 slices, (i) 5.7 ± 0.4 , *n* = 1660 cells, 16 slices, (d) 5.4 ± 0.3 , *n* = 762 cells, 9 slices; 300–400 μ m: (v) 1.4 ± 0.8 , *n* = 133 cells, 2 slices, (i) 3.5 ± 0.3 , *n* = 1376 cells, 16 slices, (d) 4.3 ± 0.4 , *n* = 557 cells, 8 slices; 400–500 μ m: (i) 1.2 ± 0.2 , *n* = 563 cells, 10 slices, (d) 1.3 ± 0.3 , *n* = 205 cells, 5 slices.

Table 3. Summary of terms in the final chosen model for PaS neuron counts

	Coefficient	z	p
Stain (NeuN)	2.185	6.518	0.000
Stain (VGAT)	−0.631	−2.076	0.038
Stain (WFS1)	−1.152	−3.621	0.000
Depth:NeuN	2.394	6.871	0.000
Depth:VGAT	−0.985	−4.071	0.000
Depth:WFS1	0.615	2.427	0.015
Depth ² :NeuN	−0.658	−5.905	0.000
Depth ² :VGAT	0.177	4.323	0.000
Depth ² :WFS1	−0.117	−2.721	0.007
Depth ³	0.053	4.441	0.000

Table 4. Summary of terms in final model of neuron counts in PaS, including dorsoventral axis

	Coefficient	z	p
Stain (NeuN):slice position (dorsal)	0.686	1.712	0.087
Stain (VGAT):slice position (dorsal)	0.653	1.689	0.091
Stain (WFS1):slice position (dorsal)	−0.483	−1.196	0.232
Stain (NeuN):slice position (intermediate)	0.902	2.802	0.005
Stain (VGAT):slice position (intermediate)	−0.876	−2.544	0.011
Stain (WFS1):slice position (intermediate)	−0.720	−2.007	0.045
Stain (NeuN):slice position (ventral)	2.462	4.300	0.000
Stain (VGAT):slice position (ventral)	−1.990	−3.304	0.001
Stain (WFS1):slice position (ventral)	−0.904	−1.473	0.141
Depth:NeuN:dorsal	3.037	9.364	0.000
Depth:VGAT:dorsal	−1.560	−6.648	0.000
Depth:WFS1:dorsal	0.431	1.752	0.080
Depth:NeuN:intermediate	−0.174	−1.682	0.093
Depth:VGAT:intermediate	0.228	2.051	0.040
Depth:WFS1:intermediate	0.169	1.463	0.144
Depth:NeuN:ventral	−0.903	−2.768	0.006
Depth:VGAT:ventral	0.951	2.824	0.005
Depth:WFS1:ventral	0.371	1.092	0.275
Depth ² :NeuN	−0.746	−7.573	0.000
Depth ² :VGAT	0.235	6.314	0.000
Depth ² :WFS1	−0.109	−2.793	0.005
Depth ³	0.056	5.433	0.000

tures including the shape and location of the PaS were assigned to either the top, central, or bottom third of the PaS structure, corresponding to dorsal, intermediate, and ventral portions, respectively. Subsequently, we added DV location as a new factor in our GLM and found that it was also a significant factor (cell count $\sim$ stain*depth*DV location + stain*depth² + depth³; Fig. 2F; Table 4). Thus, we modeled estimated cell densities for WFS1 and VGAT-Venus at our three locations. In the superficial PaS (0–100 μ m from the pia), we found higher densities of both WFS1 and VGAT-Venus (Fig. 2G,H) in the ventral portion compared with intermediate and dorsal portions. WFS1 expression reached its peak density between 100 and 200 μ m from the pia in the ventral PaS, compared with 200–300 μ m in the intermediate and dorsal portions. WFS1 density in the ventral portion then decreased and became significantly lower than in intermediate and dorsal portions at deeper depths. Furthermore, the shape of the PaS changes along the DV axis; and in ventral slices, we were unable to unequivocally distinguish the PaS beyond 400 μ m from the pia. Thus, no counts were made in ventral slices at the 400–500 μ m bin. VGAT-Venus remained higher in the ventral PaS than intermediate and dorsal PaS across all depths. To examine what these results might mean for the balance of putative excitatory and inhibitory populations, we next looked at the ratio of WFS1 to VGAT-Venus cells in slices where both markers were counted (Fig.

2I). For each counting box, the number of WFS1 cells was divided by the number of VGAT-Venus cells, giving us an indication of the putative excitatory to inhibitory cell ratio. In all regions, the ratio was >1 , indicating that WFS1 cells outnumber VGAT-Venus cells throughout the PaS. In the most superficial region, the ventral WFS1:VGAT-Venus ratio was higher than intermediate or dorsal ratios. However, at all other depths, the ventral PaS showed a lower ratio of WFS1:VGAT-Venus than intermediate or dorsal PaS. Moreover, the peak ratio was much higher in dorsal and intermediate portions than in the ventral PaS, again indicating that the ventral PaS has a greater inhibitory contribution.

Different inhibitory markers exhibit differential expression profiles in the PaS

To examine the inhibitory population of neurons in the PaS in more detail, further immunostaining was performed using several different inhibitory markers (Fig. 3A), and cell counts were made for each marker (Fig. 3B). By using the VGAT-Venus transgenic mouse line, we were able to determine the relative proportion of each marker across the interneuron population (Fig. 3C). We applied the same approach as with WFS1 and VGAT-Venus density measurements and modeled our count data with a GLM (Fig. 3D; Table 5). Again, we found that both stain and depth and their interaction were good predictors of cell density, which could be described with the same expression. We observed several distinct distributions in the inhibitory markers tested (Fig. 3E). Notably, we found that, in the most superficial region of the PaS (0–100 μ m from pial surface), reelin showed a higher density above all other markers, with the exception of vasoactive intestinal peptide (VIP), where the difference was not significant (median, 25th and 75th percentile density, at 0–100 μ m from the pia for reelin, 1600, 1200–2000; calretinin, 200, 0–400; parvalbumin (PV), 100, 0–350; somatostatin (SOM), 0, 0–100; VIP, 400, 300–600; calbindin, 0, 0–200; cholecystokinin (CCK), 0, 0–0; neuropeptide Y (NPY), 0, 0–0 cells/mm³). Reelin was also at its highest density in this region, before showing relatively constant, lower density levels throughout the rest of the PaS. In contrast, several other markers, including calbindin, PV, and SOM, showed the inverse pattern, starting with very low levels in the superficial PaS and having the highest density counts throughout the middle and deepest portions of the PaS. Meanwhile, NPY, VIP, calretinin, and CCK did not show such distinct distributions, with relatively uniform, low expression levels throughout the superficial to deep axis of the PaS. However, VIP and calretinin did have significantly higher counts than CCK and NPY in the area spanning 200–300 μ m from the pial surface.

Immunolabeling for different inhibitory markers was always performed using pairs of antibodies, in slices from VGAT-Venus transgenic mice. Thus, we were also able to determine overlap between certain pairs of inhibitory markers (Fig. 3F). We found that both PV and SOM overlapped partially with calbindin, explaining, in part, the similarities in density distribution patterns seen across these markers. We also observed some coexpression between reelin and both SOM and calbindin. Furthermore, we found that a small percentage of reelin-positive neurons were not VGAT-Venus-positive. These neurons were not included when calculating the total percentage of reelin in the overall VGAT-Venus population. We did not find any overlap between WFS1 and any of the inhibitory markers with which it was coimmunostained for (calretinin, PV, calbindin, or reelin). This work reveals that there is a small population of noninhibitory reelin-positive neurons, and that these cells are, at least molecularly, distinct from the main pyramidal class in the PaS.

Figure 3. Different inhibitory markers within the VGAT-Venus population. **A**, Example images of slices immunostained with each of the molecular markers. Dashed yellow line indicates PaS boundaries. Scale bars, 100 μm. **B**, Boxplots of cell counts for each marker at different depths of the PaS, measured as distance from pia. Horizontal black line indicates median. Colored box

Parasubicular neurons can be classified into three clusters based on their electrophysiological properties

To characterize the electrophysiological features of neurons in the PaS, we made whole-cell recordings in acute brain slices from 391 neurons within this brain region. Following recordings, slices were immunostained to enable visualization of the recorded neurons, which were filled with biocytin during recording, as well as to label for potential molecular markers. We performed unsupervised cluster analysis and principal component analysis on our electrophysiological data to determine whether we could identify different cell groups based on their intrinsic and AP firing properties. In addition to electrophysiological parameters, for a subset of cells, we were able to recover *post hoc* immunohistochemical labeling of molecular markers, which we matched up to cells after clustering (Fig. 4A). To assess our clustering, we calculated the gap statistic (Tibshirani et al., 2001) to look at inter-cluster variance, and the CH index to examine the ratio of intercluster to intra-cluster variability. Both measures were calculated for up to 10 clusters (Fig. 4B). The CH index decreased as the number of clusters increased, indicating that clusters became less compact the more clusters there were. Our gap plot indicated four to be the optimal number of clusters. Cutting our dendrogram at the level of four clusters produced one cluster containing two cells. Thus, we merged this cluster

represents interquartile range. Whiskers indicate minimum and maximum points. Colors are the same as in **A**. Numbers above each boxplot indicate number of slices data were gathered from at each distance interval, for each marker. **C**, Proportion of cells colabeled with each inhibitory marker and VGAT-Venus, as a percentage of total VGAT-Venus cells: calretinin: 79 of 667 cells = 12%, $n = 13$ slices, 2 animals; PV: 181 of 842 cells = 21%, $n = 14$ slices, 4 animals; SOM: 83 of 397 cells = 21%, $n = 7$ slices, 4 animals; VIP: 37 of 242 cells = 15%, $n = 4$ slices, 2 animals; calbindin: 531 of 1407 cells = 38%, $n = 27$ slices, 2 animals; reelin: 17%, $n = 15$ slices, 4 animals; CCK: 10 of 583 cells = 2%, $n = 7$ slices, 4 animals; NPY: 10 of 252 cells = 4%, $n = 5$ slices, 2 animals. **D**, Comparison of AIC (gray) and deviance (black) for each of the tested models for inhibitory subclass density distributions. M1: count ~ depth*stain; M2: M1 + depth²; M3: M2 + depth³; M4: M2 + stain*depth²; M5: M4 + depth³; M6: M5 + stain*depth³, where an asterisk represents the inclusion of both predictors as well as their interaction. p values indicate outcome of likelihood ratio test performed between nested models. **E**, Predicted density distributions of the different inhibitory markers across the depth of the PaS, using the chosen model (Model 5). Shaded area represents 95% CIs. Solid line indicates mean. **F**, Probability matrix for coexpression of different inhibitory markers tested.

Table 5. Summary of terms in final model of inhibitory neuron counts in the PaS

	Coefficient	<i>z</i>	<i>p</i>
Stain (calbindin)	−4.347	−4.787	0.000
Stain (calretinin)	1.630	0.964	0.335
Stain (CCK)	4.152	1.347	0.178
Stain (NPY)	−5.736	−1.435	0.151
Stain (PV)	0.611	0.372	0.710
Stain (reelin)	9.282	6.858	0.000
Stain (SOM)	−0.869	−0.363	0.717
Stain (VIP)	3.572	1.438	0.151
Depth:calbindin	5.994	5.276	0.000
Depth:calretinin	−2.053	−0.951	0.342
Depth:CCK	−6.558	−1.574	0.115
Depth:NPY	6.740	1.343	0.179
Depth:PV	−1.012	−0.484	0.629
Depth:reelin	−9.988	−5.644	0.000
Depth:SOM	−0.096	−0.033	0.974
Depth:VIP	−3.237	−0.984	0.325
Depth ² :calbindin	−1.799	−4.307	0.000
Depth ² :calretinin	0.354	0.441	0.659
Depth ² :CCK	1.689	1.078	0.281
Depth ² :NPY	−3.108	−1.628	0.104
Depth ² :PV	0.298	0.379	0.704
Depth ² :reelin	3.033	4.566	0.000
Depth ² :SOM	0.084	0.078	0.938
Depth ² :VIP	0.653	0.522	0.602
Depth ³ :calbindin	0.173	3.734	0.000
Depth ³ :calretinin	−0.015	−0.169	0.8657
Depth ³ :CCK	−0.126	−0.726	0.4679
Depth ³ :NPY	0.374	1.733	0.0831
Depth ³ :PV	−0.025	−0.286	0.7752
Depth ³ :reelin	−0.289	−3.879	0.0001
Depth ³ :SOM	−0.009	−0.072	0.9429
Depth ³ :VIP	−0.047	−0.328	0.7430

with its closest neighbor cluster and looked at the resulting three clusters (Fig. 4C). The first cluster (55 of 391 cells) contained many inhibitory labeled neurons. In 10 cells, PV immunostaining was recovered; and in a further 7 cells, VGAT-Venus labeling was confirmed. Two cells were identified as reelin-positive. Cluster 2 (52 of 391 cells) contained 17 reelin-positive cells, as well as a further 3 VGAT-Venus-positive neurons and 2 WFS1-labeled neurons. Combined with our previous finding that, in the PaS, reelin is predominantly an inhibitory marker, we propose that Cluster 2 may also represent a putatively inhibitory cluster of neurons. Cluster 3 represented more than two-thirds of all recorded neurons (284 of 391 cells). More than half of Cluster 3 cells (155 of 284) were found to be WFS1-positive. Given the lack of colocalization between WFS1 and VGAT-Venus seen earlier, we propose Cluster 3 to be a putatively excitatory group of neurons.

In 301 of our recorded cells, we were able to successfully recover the biocytin labeling and identify the recorded neuron. For these cells, we measured the minimal distance to the pial surface from the cell soma (Fig. 4A,D) and binned them into 100 μ m intervals matching those used in our immunohistochemistry experiments (Fig. 4E). We looked at the proportion of putative inhibitory and excitatory neurons recorded at different depths of the PaS by combining Cluster 1 and Cluster 2 cells to represent putative inhibitory neurons. We found the greatest proportion of putative inhibitory neurons to be in the range of 300–400 μ m from the pia (38% putative inhibitory, 62% putative excitatory), followed by the most superficial region, 0–100 μ m from the pia (33% putative inhibitory, 67% putative excitatory). In the deep-

est portion (>400 μ m from pia), we found only Cluster 3, or putative excitatory neurons. However, overall only 5 cells were recorded in this region due to difficulties in targeting accurately the deep region of this structure. In the central regions (100–300 μ m), we saw high levels of putative excitatory neurons (100–200 μ m: 81% putative excitatory; 200–300 μ m: 70% putative excitatory) reflecting similarities with our immunohistochemistry data. We next looked at the distribution of each cluster across the measured depths (Fig. 4G–I). We found that Cluster 1 cells were predominantly found in the central region of the PaS (37% of all Cluster 1 cells in each 100–200 μ m and 200–300 μ m bins). Meanwhile, the majority of Cluster 2 cells were found in the most superficial region (0–100 μ m: 57% all measured Cluster 2 cells). Cluster 3 cells were found to be most abundant in the central portions of the PaS (100–200 μ m: 43% of all Cluster 3 cells; 200–300 μ m: 25% of all Cluster 3 cells).

Each cluster could be distinguished by several electrophysiological features that separated it from the remaining two clusters (Table 6). To identify the features that most strongly typified each class, we calculated a measure of effect size (ES) for each parameter. Since many of our parameters did not follow a normal distribution, we used Cliff's δ to quantify the differences between clusters (Cliff, 1996) (Table 6). Several features were found to be strongly different across all three clusters. These include the ratio of AP rise and fall rates (Fig. 5A; median, IQR dV/dt ratio C1: 1.21, 1.1–1.32; C2: 1.94, 1.79–2.13; C3: 2.57, 2.17–3.2; $p < 0.001$ for all comparison pairs using Dunn's test; ES C1 vs C2: −0.85; C1 vs C3: −0.99; C2 vs C3: −0.61), minimal interspike intervals (Fig. 5B; median, IQR min interspike interval C1: 10, 6.9–14.3 ms; C2: 18.1, 14.9–22.6 ms; C3: 44.7, 34.5–54.9 ms; C1 vs C1, $p = 0.04$, ES: −0.67; $p < 0.001$ for all other comparison pairs using Dunn's test; ES C1 vs C3: −0.95; C2 vs C3: −0.89) and input resistance (Fig. 5C; median, IQR input resistance C1: 62.7, 46–76 M Ω ; C2: 152.7, 120.6–230.1 M Ω ; C3: 116.6, 89.4–149.3 M Ω ; $p < 0.001$ for all comparison pairs using Dunn's test, ES C1 vs C2: −0.83; C1 vs C3: −0.67; C2 vs C3: 0.44). Furthermore, the three clusters all showed distinct input–output curves (Fig. 5D–Diii; median, IQR input–output slope C1: 0.8, 0.7–1.2; C2: 0.7, 0.5–0.9; C3: 0.2, 0.2–0.3; $p < 0.001$ for all comparisons using Dunn's test; ES C1 vs C2: 0.36; C1 vs C3: 0.97; C2 vs C3: 0.78). Cluster 1 cells showed later activation, reflected in their high rheobase values, whereas Clusters 2 and 3 showed much earlier activation (median, IQR rheobase C1: 358, 249–440 pA; C2: 98, 46–152 pA; C3: 109, 71–148 pA; C2 vs C3, $p = 0.2$; $p < 0.001$ for all other comparison pairs using Dunn's test; ES C1 vs C2: 0.84; C1 vs C3: 0.75). Inactivation of some neurons was seen upon injection of high current values and was characterized by the failure of neurons to fire APs that reversed >0 mV. This inactivation followed the same pattern as activation, occurring early on in Cluster 2 cells and later or not at all in Cluster 1 cells. Other features were highly indicative of specific clusters. Cluster 1 cells showed high firing frequencies (Fig. 5D,E; median, IQR relative firing frequency C1: 223, 181–288 Hz; C2: 94, 71–114 Hz; C3: 56, 42–76 Hz; $p < 0.001$ for all comparisons using Dunn's test; ES C1 vs C2: 0.94; C1 vs C3: 0.99; C2 vs C3: 0.57) and a short AP half-width (Fig. 5F; median, IQR AP half-width C1: 0.24, 0.18–0.28 ms; C2: 0.56, 0.49–0.67 ms; C3: 0.67, 0.56–0.77 ms; $p < 0.001$ for all comparison pairs using Dunn's test; ES C1 vs C2: −0.99; C1 vs C3: −1; C2 vs C3: −0.36). Cluster 3 cells were typified by a large AP height (Fig. 5F; median, IQR AP height C1: 61.8, 51–71.7 mV; C2: 56.4, 50.1–67.5 mV; C3: 70.4, 63.5–77.3 mV; C1 vs C2, $p = 0.12$; $p < 0.001$ for all other comparison pairs using Dunn's test; ES C1 vs C3: −0.37; C2 vs C3: −0.55) and the presence of a sag

Figure 4. Clustering of PaS neurons. **A**, Unsupervised hierarchical clustering (Ward's method) and resulting dendrogram displaying the three clusters of cells in PaS. Heatmap represents z score for each parameter used in cluster analysis, for each cell. Right, Horizontal colored bars represent *post hoc* immunostaining in recovered cells (orange represents PV; green represents VGAT-Venus; blue represents reelin; gray represents WFS1), and minimal distance to the pia of recovered cells. For both, white represents no recovered metric. **B**, Gap statistic and CH score for up to 10 clusters. For gap statistic, mean $\pm$ SD for 500 computations for each cluster size are plotted. **C**, Scatter plot of the first two principal components. Each point represents one cell: red represents cells in Cluster 1; blue represents cells in Cluster 2; gray represents cells in Cluster 3. **D**, Image showing method for measuring minimal distance to pia. A circle was drawn centered over the cell soma until it touched any part of the pia within the PaS boundary. The radius of this circle was then taken as the minimal distance to pia measure. **E**, Number of cells from each cluster recorded at each bin depth. Colors represent clusters as described for **C**. **F**, Percentage of cells belonging to putative excitatory (gray, Cluster 3) or inhibitory (green, Clusters 1 and 2) at each bin depth from pia. **G**, Percentage of cells from Cluster 1 recorded from each bin depth. **H**, Percentage of cells from Cluster 2 recorded from each bin depth. **I**, Percentage of cells from Cluster 3 recorded from each bin depth.

Table 6. Passive and active properties of the three clusters of cells found in PaS^a

	Cluster 1 (<i>n</i> = 55 cells in 52 slices, 41 animals)	Cluster 2 (<i>n</i> = 52 cells in 47 slices, 28 animals)	Cluster 3 (<i>n</i> = 284 cells in 204 slices, 94 animals)	<i>p</i> (effect size)		
				C1 versus C2	C1 versus C3	C2 versus C3
<i>V_m</i> (mV)	−68.4, −72.9 to −64.6	−69.9, −72.8 to −66.4	−69.1, −72.2 to −64.4	—	—	—
Time constant (ms)	5.4, 4.3 to 10.4	7.7, 5.3 to 14.7	15.9, 10.3 to 22.1	0.045 (−0.24)	<0.001 (−0.7)	<0.001 (−0.49)
Theta frequency (Hz)	5, 3.3 to 7.5	5, 3.3 to 6.7	3.3, 3.3 to 5	0.312	0.016 (0.21)	0.235
AP threshold (mV)	−37.5, −40.8 to −34.4	−35.1, −37.4 to −31	−34.9, −37 to −32.7	<0.001 (−0.34)	0.002 (−0.31)	0.630
Onset latency (ms)	118.9, 13.2 to 207	32.8, 15.8 to 60.2	132, 83.1 to 178.6	<0.001 (0.28)	0.040 (−0.12)	<0.001 (−0.8)
Maximum dV/dt (<i>V</i> ·s ^{−1})	398, 299 to 605	211, 173 to 273	313, 237 to 370	<0.001 (0.91)	<0.001 (0.58)	<0.001 (−0.55)
Minimum dV/dt (<i>V</i> ·s ^{−1})	−323, −517 to −248	−120, −141 to −91	−110, −135 to −93	<0.001 (−0.99)	<0.001 (−0.99)	0.412
AP2–AP3 interval (ms)	18.4, 9.6 to 66.3	22.5, 15.7 to 60.2	63.9, 51.3 to 76.3	0.147	<0.001 (−0.47)	<0.001 (−0.73)
AP9–AP10 interval (ms)	17, 10 to 39.1	24.4, 20.6 to 32.3	55.7, 43.5 to 69.8	0.922	<0.001 (−0.69)	<0.001 (−0.79)
Adaptation index	1.02, 0.74 to 1.33	1.09, 0.83 to 1.32	0.9, 0.81 to 1.06	0.434	0.045 (0.18)	0.005 (0.28)
AHP (mV)	25.1, 22.5 to 27.6	18.3, 14.9 to 21.2	16.3, 13.9 to 18.8	<0.001 (0.75)	<0.001 (0.85)	0.003 (0.28)
Medium AHP (mV)	−0.005, −0.007 to −0.003	−0.01, −0.018 to 0.004	−0.022, −0.035 to −0.01	0.015 (0.36)	<0.001 (0.61)	<0.001 (0.37)

^aValues are reported as median and IQR. *p* values from *post hoc* Dunn's test corrected for multiple comparisons using false discovery rate correction. Recordings were made from a total of 262 slices from 98 animals. ES was calculated as Cliff's δ for significantly different pairs.

potential in response to negative current injection (Fig. 5G; median, IQR sag ratio C1: 0.9, 0.87–0.93; C2: 0.92, 0.9–0.94; C3: 0.88, 0.83–0.91; C1 v C2, *p* = 0.025, ES: −0.23; C1 vs C3, *p* = 0.007, ES: 0.24; C2 v C3, *p* < 0.001, ES: 0.49). It has been previously reported that PaS neurons exhibit membrane oscillations within the theta range (Glasgow and Chapman, 2008). Thus, we checked subthreshold membrane depolarizations for activity in the theta frequency band. We found prominent subthreshold theta-range membrane activity in Cluster 2 and 3 neurons, but not in Cluster 1 neurons (Fig. 5H–J; median, IQR subthreshold theta power C1: 2×10^5 , 1.2×10^5 to 6.3×10^5 mV²; C2: 5.8×10^5 , 2.7×10^5 to 2.2×10^6 mV²; C3: 4.6×10^5 , 2.3×10^5 to 1.2×10^6 mV²; *p* < 0.001 for all comparison pairs using Dunn's test; ES C1 vs C2: −0.39; C1 vs C3: −0.32; C2 vs C3: 0.12).

In addition to electrophysiological properties, we also saw morphological differences between cells in the three clusters. Cluster 1 was the only cluster in which PV staining was observed (Fig. 6A). Cells in this cluster tended to show morphologies that covered a large area of the PaS (Fig. 6B,C). In contrast, Cluster 2 cells often showed small morphologies (Fig. 6D,E), also reflected in their low membrane capacitance values (median, IQR *C_m* C1: 89.9, 70.6–133 pF; C2: 46.8, 33.1–87 pF; C3: 137.7, 103.2–189.8 pF; *p* < 0.001 for all comparison pairs using Dunn's test; ES C1 vs C2: 0.55; C1 vs C3: −0.35; C2 vs C3: −0.76). Cluster 3 cells showed typical pyramidal morphology with apical dendrites extending to the pial surface. Axons from Cluster 3 cells were seen projecting toward the alveus (Fig. 6F,G) as well as the MEC (Fig. 6H,I).

Our initial cluster analysis suggested the possible presence of further subgroups of cells, in particular within the principal neurons comprising Cluster 3. Therefore, we reran our clustering algorithm on Cluster 3, after first removing any VGAT-positive cells to leave us with a putatively pure excitatory set of cells (Fig. 7A, *n* = 280 cells). The CH index decreased with increasing numbers of clusters, again indicating the presence of fewer, tighter clusters (Fig. 7B). Our gap plot indicated 3 to be the optimal number of clusters (Fig. 7B). Moreover, the largest decrease in Euclidean distance was also seen at this stage (Fig. 7C). Thus, we cut the dendrogram at the level for three clusters and looked at these groups in more detail. All three groups contained a high proportion of WFS1, and reelin was also seen in all three groups (C3a total cluster size: 115 cells; 62% WFS1, 6% reelin; C3b: 75 cells; 45% WFS1, 1% reelin; C3c total cluster size: 90 cells; 56% WFS1, 3% reelin). While all of these cells are putatively excitatory pyramidal neurons, the three groups differed in a number of parameters (Table 7). Neurons in C3a were characterized by a

short AP height and high threshold (Fig. 7D,E; median, IQR AP height in C3a, 62.2, 55.6–67.8 mV; C3b: 71.9, 69.4–77 mV; C3c: 78.5, 73.3–82.8 mV; C3a vs C3b, *p* < 0.001, ES: −0.73; C3a vs C3c, *p* < 0.001, ES: −0.81; C3b vs C3c, *p* = 0.003, ES: 0.4; Dunn's test and Cliff's δ ; median, IQR AP threshold C3a: −33.6, −34.8 to −30.9 mV; C3b: −35.8, −37.5 to −34.5 mV; C3c: −36.3, −38.5 to −34.3; C3a vs C3b, *p* < 0.001, ES: 0.51; C3a vs C3c, *p* < 0.001, ES: 0.54; C3b vs C3c, *p* = 0.489). C3b neurons were typified by a short AP half-width (Fig. 7F; median, IQR AP half-width C3a, 0.73, 0.63–0.83 ms; C3b, 0.55, 0.5–0.58 ms; C3c, 0.73, 0.65–0.81 ms; C3a vs C3b, *p* < 0.001, ES: 0.8; C3a vs C3c, *p* = 0.567; C3b vs C3c, *p* < 0.001, ES: 0.84), a large AHP (median, IQR AHP C3a: 15.7, 13.9–18.4 mV; C3b: 18.2, 16.4–20.5 mV; C3c: 14.6, 12.6–17.4 mV; C3a vs C3b, *p* < 0.001, ES: −0.39; C3a vs C3c, *p* = 0.025, ES: 0.2; C3b vs C3c, *p* < 0.001, ES: −0.53), and a low input resistance (median, IQR input resistance C3a: 131, 110–165 M Ω ; C3b: 91, 76–107 M Ω ; C3c: 124, 94–159 M Ω ; C3a vs C3b, *p* < 0.001, ES: 0.63; C3a vs C3c, *p* = 0.152; C3b vs C3c, *p* < 0.001, ES: 0.46). Cells in C3c showed the lowest firing frequencies (median, IQR relative firing frequency C3a: 60, 49–76 Hz; C3b, 75, 63–86 Hz; C3c, 41, 29–49 Hz; C3a vs C3b, *p* = 0.002, ES: −0.31; C3a vs C3c, *p* < 0.001, ES: 0.62; C3b vs C3c, *p* < 0.001, ES: −0.81), the shortest onset latency to first AP (median, IQR onset C3a: 141, 97–191 ms; C3b: 160, 116–210 ms; C3c: 95, 61–135 ms; C3a vs C3b, *p* = 0.06; C3a vs C3c, *p* < 0.001, ES: 0.34; C3b vs C3c, *p* < 0.001, ES: −0.5), and the highest dV/dt ratio (median, IQR dV/dt ratio C3a: 2.3, 2.0–2.6; C3b: 2.4, 2.1–2.6; C3c: 3.6, 3.1–4.1; C3a vs C3b, *p* = 0.286; C3a vs C3c, *p* < 0.001, ES: −0.84; C3b vs C3c, *p* < 0.001, ES: 0.81). We checked the location of cells from each of the three groups to determine whether they might be differentially distributed throughout the PaS (Fig. 7F). However, cells from each group were found in each of our binned distances from the pia, and we saw no statistical difference between groups (Kruskal–Wallis test, *p* = 0.316). Thus, these subgroups of pyramidal neurons do not appear to occupy distinct regions but rather intermingle throughout the PaS.

Discussion

The PaS is home to several functionally specialized cell types important for the integration of spatial information, including head-direction, border, and grid cells (Taube, 1995; Boccara et al., 2010). How these functional cell types interact with one another will be key in detangling how the spatial network operates. Such connectivity studies have been performed in the MEC (Couey et al., 2013; Fuchs et al., 2016; Winterer et al.,

Figure 5. Electrophysiology of parasubicular neurons. **A**, Scatter plot of minimum versus maximum dV/dt for each cell. **B**, **C**, Boxplots for minimal interspike interval and input resistance. Horizontal line indicates median. Colored boxes represent interquartile range. Whiskers indicate minimum and maximum points. **D**, Top, Input–output function of Cluster 1 (**Di**), Cluster 2 (**Dii**), and Cluster 3 (**Diii**) cells. Data are mean $\pm$ SEM. Bottom, Percentage of cells activated (green) and inactivated (black) at each current injection step, and total number of cells (black circles) included in mean calculation at each step (i.e., total number of preactivation plus activated neurons). **E**, Typical firing pattern of a cell from Cluster 1 (**Ei**), Cluster 2 (**Eii**), and Cluster 3 (**Eiii**) at the first current step to induce an AP, with increased current to induce higher firing frequencies and in response to a negative current injection. Bottom, Schematic depicts current injection. **F**, Zoom-in of the first AP from example cell in each cluster. Waveforms are aligned at threshold. **G**, Zoom-in of the voltage response to a hyperpolarizing current injection in one example cell from each cluster. **H**, FFT of subthreshold traces shown in **J**. Shaded area represents theta frequency range. **I**, Boxplot of maximal theta power for each cluster. Black horizontal line indicates median. Shaded box represents interquartile range. Whiskers indicate total range. **J**, Raw subthreshold membrane potential trace from an exemplary cell from each cluster.

2017) and the PrS (Peng et al., 2017). However, our knowledge of the basic cell types in the PaS is poor compared with other parahippocampal structures. We performed immunohistochemistry and electrophysiology to determine the basic neuronal composition of the PaS. Cell counts revealed differential

expression of molecularly distinct neurons across both superficial to deep and DV axes. Unsupervised clustering of electrophysiological parameters identified three major groups of neurons, which showed a number of parallels with our histological data.

Figure 6. Morphology of the PaS neuronal population. **Ai**, Slice containing three recorded neurons, immunolabeled with WFS1 and PV after recording. **Aii**, Closeup of somata showing biocytin, WFS1, and PV labeling. Two cells (indicated by white arrows) are positive for WFS1 and reside in Cluster 3, whereas the third cell (indicated by yellow arrow) is PV-positive and classified as Cluster 1. **B**, Maximum projection confocal image of a biocytin-filled cell from Cluster 1, stained for WFS1 (white) to identify the PaS. Right, Closeup of cell soma showing immunolabeling of biocytin, WFS1, and reelin. Scale bars, 100 and 10 μm . **C**, Morphological reconstruction of cell in **B**. Black represents dendrites and soma. Red represents axon. Dotted line indicates outline of PaS, as determined by WFS1 labeling. Scale bar, 100 μm . **D**, Maximum projection confocal image of reelin-positive Cluster 2 neuron. Right, Closeup of cell soma and immunolabeling of biocytin, WFS1, and reelin. Scale bars, 100 and 10 μm . **E**, Morphological reconstruction of cell in **D**. Black represents dendrites and soma. Red represents axon. Scale bar, 100 μm . **F**, **H**, Maximum projection confocal images of biocytin-filled cells in Cluster 3. **F**, Right, Closeup of soma showing

Lying at the interface between the three-layered hippocampus and six-layered cortex, the PaS has been variously described as a three- and six-layered structure (Funahashi and Stewart, 1997; Mulders et al., 1997; Glasgow and Chapman, 2007; Boccara et al., 2010; Buralossio et al., 2011; Tang et al., 2016). We analyzed neuronal markers along the superficial to deep axis to shed more light on the issue. Overall cell density was lowest in the most superficial region, consistent with L1 of the neocortex across numerous species (Gabbott and Somogyi, 1986; Amaral et al., 1987; Meyer et al., 2010). This region was enriched with inhibitory neurons, especially reelin-expressing, in line with reports that L1 is interneuron rich, and specifically the 5HT3-receptor interneuron subclass of which reelin is a member (Tremblay et al., 2016; Keller et al., 2018). Cell density reached its peak in the central PaS, before decreasing again in the deepest region. The MEC exhibits a cell sparse lamina densicans, which dissects the deep and superficial layers (Canto et al., 2008). In the majority of our slices, the PaS was no longer discernible from its neighboring structures beyond 500 μm , thus suggesting that the low-density region is the deepest layer of the PaS. In this deepest measured region, we saw the peak density of VGAT-Venus, and in particular PV and SOM. Similarly, in the cortex, these markers exhibit their peak densities in the deepest layers (Keller et al., 2018). Thus, it appears that the cell sparse layer and PV/SOM rich deep layers merge in the PaS. It is, however, possible that the size of our measuring bins precludes us from definitively distinguishing between different deep layers.

The hippocampus, subiculum, and MEC have all been reported to show differences across the DV axis, from gene expression and intrinsic properties to projection targets and functional separations (Moser et al., 1995; Risold and Swanson, 1996; Brun et al., 2008; Giocomo et al., 2011; Kheirbek et al., 2013; Cembrowski et al., 2016). To determine whether the PaS also exhibits DV differences, we looked at the ratio of putative excitatory to inhibitory neuron markers across this axis. We found a higher proportion of inhibitory neurons in the ven-

← biocytin and WFS1 labeling. Scale bars, 100 and 10 μm . **G**, **I**, Morphological reconstructions of cells in **F** and **H**, showing soma and dendrites in black and axon in red. Dashed lines outline the borders of the PaS. Scale bar, 100 μm .

Figure 7. Clustering of pyramidal neurons in the PaS. **A**, Dendrogram for hierarchical clustering of Cluster 3 pyramidal neurons. Vertical lines indicate *post hoc* immunolabeling. Gray represents WFS1. Blue represents reelin. White represents no data. Red dotted line indicates position of dendrogram cutting. **B**, Gap statistic and CH index for up to 10 clusters. For gap statistic, mean $\pm$ SD for 500 computations are plotted for each number of clusters. **C**, Plot showing Euclidean distance at each cluster stage. **D**, Example firing patterns and response to negative current injection in Cluster 3a (**Di**), Cluster 3b (**Dii**), and Cluster 3c (**Diii**) neurons. **E**, Zoom-in of the first AP from cells in **D**. **F**, Distribution of locations for cells in Cluster 3a (black), Cluster 3b (dark gray), and Cluster 3c (light gray).

Table 7. Properties of subclasses of pyramidal neurons in the PaS^a

	Cluster 3a (<i>n</i> = 115 cells in 91 slices, 56 animals)	Cluster 3b (<i>n</i> = 75 cells in 64 slices, 42 animals)	Cluster 3c (<i>n</i> = 90 cells in 74 slices, 52 animals)	<i>p</i> (effect size)		
				C3a versus C3b	C3a versus C3c	C3b versus C3c
<i>V_m</i> (mV)	−69.5, −72.3 to −65.2	−71, −73.2 to −67.9	−66.7, −71.1 to −61.8	0.03 (0.19)	0.03 (−0.18)	<0.001 (0.36)
Sag ratio	0.89, 0.86 to 0.92	0.86, 0.83 to 0.91	0.87, 0.78 to 0.91	0.012 (0.25)	0.001 (0.28)	0.461
Time constant (ms)	16, 11.4 to 20.9	11, 8.8 to 17.3	21.1, 14.6 to 26.8	0.001 (0.31)	<0.001 (−0.31)	<0.001 (0.55)
Membrane capacitance (pF)	119.9, 89.1 to 187.7	126.4, 108.1 to 195.5	163.8, 125.7 to 207.4	0.341	<0.001 (−0.32)	0.007 (0.28)
Theta power (mV ²)	4.4×10^5 , 2.3×10^5 to 1.2×10^6	3.8×10^5 , 2×10^5 to 8.9×10^5	5.3×10^5 , 2.6×10^5 to 1.7×10^6	—	—	—
Rheobase (pA)	96, 70 to 132	141, 115 to 180	96, 40 to 140	<0.001 (−0.45)	0.892	<0.001 (0.39)
Maximum dV/dt (V·s ^{−1})	232, 182 to 275	357, 308 to 390	358, 314 to 407	<0.001 (−0.82)	<0.001 (−0.77)	0.885
Minimum dV/dt (V·s ^{−1})	−99, −116 to −81	−146, −159 to −131	−103, −112 to −89	<0.001 (0.85)	0.397	<0.001 (0.84)
AP2–AP3 interval (ms)	63, 51.2 to 76	63.9, 53.8 to 74.4	65.2, 50.1 to 77.8	—	—	—
AP9–AP10 interval (ms)	51.7, 40.7 to 61.2	51.7, 41.2 to 62.2	70, 55.1 to 84.4	0.634	<0.001 (−0.55)	<0.001 (0.5)
Adaptation index	0.86, 0.78 to 0.98	0.91, 0.82 to 1.03	0.95, 0.84 to 1.15	0.076	<0.001 (−0.3)	0.076
Minimum interspike interval (ms)	41.8, 32.1 to 50.7	43, 34.6 to 51.5	50.8, 37.2 to 60.9	0.418	0.001 (−0.28)	0.021 (−0.23)
Input–output slope	0.28, 0.19 to 0.4	0.24, 0.21 to 0.31	0.17, 0.13 to 0.23	0.103	<0.001 (0.55)	<0.001 (−0.46)
Medium AHP (mV)	−0.026, −0.041 to −0.012	−0.025, −0.035 to −0.016	−0.015, −0.027 to −0.007	0.714	0.003 (−0.25)	0.013 (0.26)

^aValues are reported as median and IQR. *p* values from *post hoc* Dunn's test corrected for multiple comparisons using false discovery rate correction. Recordings were made from a total of 200 slices in 94 animals. ES was calculated as Cliff's δ for significantly different pairs.

tral PaS compared with the dorsal PaS, with the exception of the most superficial region. This contrasts with work in the MEC showing a greater inhibitory drive onto L2 stellate cells in dorsal MEC compared with ventral (Beed et al., 2013). It is important to note that our findings relate only to the numbers of inhibitory neurons and may not reflect functional levels of inhibition in dorsal and ventral PaS. Thus, further experiments are required to determine the consequence of this observed difference.

We observe a notably different organization in the PaS compared with its neighboring cortex. Coexpression of specific molecular markers proved an effective way of discriminating between PaS and MEC. We observed two major differences between the two areas, and identified a previously reported intermediate re-

gion between them, which we designate to the mMEC (Fujimaru and Kosaka, 1996; Ray et al., 2017). First, in the PaS, calbindin is exclusively expressed in inhibitory neurons; whereas in the MEC, it colocalizes with WFS1 and labels the pyramids of L2 (Ray et al., 2014; Kitamura et al., 2015; Ramsden et al., 2015); and second, in the PaS, reelin is predominantly expressed in inhibitory neurons; whereas in the MEC, it labels the second population of excitatory principal cells, stellate cells (Varga et al., 2010). In contrast to the MEC, where principal cells can be divided into pyramids and stellates based on electrophysiological and morphological features (Alonso and Klink, 1993; Klink and Alonso, 1997), as well as molecular markers (Varga et al., 2010; Kitamura et al., 2014; Ray et al., 2014; Fuchs et al., 2016; Winterer et al., 2017), we find that,

while principal cells in the PaS do show some subgrouping with distinct physiological properties, they are largely labeled by a single marker, WFS1. Moreover, these subgroups were not defined by specific locations in the PaS, unlike in the MEC where L2 and L3 pyramidal neurons can be separated by V_m and input resistance, as well as the presence and lack of WFS1 expression, respectively (Winterer et al., 2017). These results again highlight the apparent lack of well-defined layers in the PaS, and indicate a subtler subdivision of principal cells in the PaS compared with the MEC. Earlier studies have referred to pyramidal and stellate populations in the PaS, although reported them to have similar electrophysiological profiles (Funahashi and Stewart, 1997). We saw a small population of putatively excitatory reelin cells, which did not exhibit a distinct profile and could potentially comprise stellate cells. Further definition of principal cell subclasses in the PaS may be established through application of additional techniques, such as morphological analysis, RNAi-seq (Cembrowski et al., 2018), or tracing studies to identify specific input or output targets.

Our cluster classification produced three major groups of neurons in the PaS, comprising of a group of fast-spiking interneurons, a group of nonfast spiking putative inhibitory neurons, and the putative principal cells of the PaS, which could be further subdivided into three groups. Cluster 1 was made up of cells with fast depolarization and repolarization rates, narrow APs, and high firing frequencies. Matching this profile, several Cluster 1 cells were PV-positive, and these neurons were primarily found in the central and deeper region of the PaS, paralleling our PV cell counts. PV interneurons have been shown to be key modulators of grid cell activity in the MEC (Buetfering et al., 2014; Miao et al., 2017) and are also implicated in induction and control of gamma oscillations (Traub et al., 1997; Cardin et al., 2009). Moreover, given the strong theta activity seen in this region (Tang et al., 2016) and the known roles of PV neurons in regulating theta activity (Stark et al., 2013; Amilhon et al., 2015) and controlling precision spike timing (Hu et al., 2014), it is likely that this cluster of neurons plays a key role in synchronizing local oscillatory activity in the PaS. Cluster 2 contained the greatest proportion of reelin-positive neurons of the three clusters. Furthermore, corresponding with our reelin cell counts, Cluster 2 neurons were most frequently recorded in the superficial PaS. Together with our finding that in the PaS reelin is predominantly an inhibitory marker, this indicates that Cluster 2 encompasses a group of superficial interneurons. Cluster 2 cells showed the strongest theta-band activity in their subthreshold membrane potential. Oscillatory activity in the membrane potential of PaS neurons has been previously described and, moreover, was found to be more prominent in superficially located neurons, including interneurons, than deeper cells (Glasgow and Chapman, 2007, 2008). Cluster 3 covered the major putative principal cell population of the PaS and contained many WFS1-positive neurons. A closer look into this cluster of cells revealed three subgroups exhibiting distinct electrophysiological features. All three of these subgroups expressed high proportions of WFS1. Small numbers of reelin cells were also found in each of these subclusters, suggesting that the non-VGAT-Venus reelin-expressing neurons do not form a physiological subclass of neurons. A previous study of PaS neurons in the rat reported subtle differences in electrophysiological properties between deep and superficial principal cells (Funahashi and Stewart, 1997). However, this was based on small sample sizes and may have also included some interneurons. While we are not able to definitively rule out that our Cluster 3 contains only excitatory neurons, the large sample size combined with the abundance of WFS1 labeling provides us with reasonable

confidence that our measurements describe well the principal cell population in the PaS. Our unbiased clustering approach did not detect physiological differences between deep and superficial principal cells but rather found that all three of our identified principal subgroups were dispersed throughout the PaS. Thus, this discrepancy may be result of a difference in species, or different experimental and analytical approaches. We saw axons of Cluster 3 cells projecting to both the MEC as well as toward the alveus, consistent with anatomical studies reporting PaS neurons projecting to both the MEC and the contralateral PaS (Köhler, 1985; van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993, 1994; Agster and Burwell, 2013). It is not known whether the same neurons project both contralaterally and ipsilaterally or whether this may be a potential distinction between different subclasses of these neurons. Cluster 3 cells showed subthreshold theta oscillations in their membrane potential; and although weaker in power than Cluster 2 cells, given the proportion of PaS neurons that this group encompasses, it is likely that these cells contribute significantly to the theta activity observed in this region.

In conclusion, we provide a detailed overview of the molecular and physiological characteristics of neurons in the PaS. We observe several distinguishing features that stand the PaS apart from the neighboring MEC. These clear differences provide future investigations with a well-defined means to avoid grouping of multiple structures or misallocation of measurements, which may potentially mask functional differences between regions. Moreover, our work defines molecular and physiological characteristics of cell types that will be key to our further understanding of the navigational and wider parahippocampal circuitry.

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