Pyramidal and Stellate Cell Specificity of Grid and Border Representations in Layer 2 of Medial Entorhinal Cortex

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http://dx.doi.org/10.1016/j.neuron.2014.11.009
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SUMMARY

In medial entorhinal cortex, layer 2 principal cells divide into pyramidal neurons (mostly calbindin positive) and dentate gyrus-projecting stellate cells (mostly calbindin negative). We juxtacellularly labeled layer 2 neurons in freely moving animals, but small sample size prevented establishing unequivocal structure-function relationships. We show, however, that spike locking to theta oscillations allows assigning unidentified extracellular recordings to pyramidal and stellate cells with ~83% and ~89% specificity, respectively. In pooled anatomically identified and theta-locking-assigned recordings, nonspatial discharges dominated, and weakly hexagonal spatial discharges and head-direction selectivity were observed in both cell types. Clear grid discharges were rare and mostly classified as pyramids (19%, 19/99 putative pyramids versus 3%, 3/94 putative stellates). Most border cells were classified as stellate (11%, 10/94 putative stellates versus 1%, 1/99 putative pyramids). Our data suggest weakly theta-locked stellate border cells provide spatial input to dentate gyrus, whereas strongly theta-locked grid discharges occur mainly in hexagonally arranged pyramidal cell patches and do not feed into dentate gyrus.

INTRODUCTION

The medial entorhinal cortex is critically involved in spatial navigation and memory. Among other functionally specialized cell types (Sargolini et al., 2006; Solstad et al., 2008; Savelli et al., 2008), it contains grid cells (Hafting et al., 2005), spatially modulated neurons which show periodic, hexagonally arranged spatial firing fields. Given the striking regularity and invariance of the grid representation, these cells are thought to be part of the brain’s coordinate system supporting spatial navigation (see Moser and Moser, 2013 for review).

Pure grid cells are primarily found in layer 2 (Boccara et al., 2010), which differs from other cortical laminae in its unique cell biology. Here the two types of principal cells, stellate and pyramidal neurons, have been described (Alonso and Klink, 1993; Germroth et al., 1989). Specifically, stellate and pyramidal neurons differ in conductances and projection patterns (Alonso and Llinás, 1989; Lingenhöhl and Finch, 1991; Klink and Alonso, 1997; Canto and Witter, 2012). Recent work indicates that stellate and pyramidal neurons can be reliably differentiated by calbindin immunoreactivity (Ray et al., 2014; Kitamura et al., 2014), and that these cells also differ in their inhibitory inputs (Varga et al., 2010). Calbindin-positive (calbindin+) cells, which are clustered and arranged in a hexagonal grid (Ray et al., 2014), have been recently shown to project to the CA1 (Kitamura et al., 2014), while calbindin-negative (calbindin–) neurons are homogeneously distributed and project primarily to the dentate gyrus (Varga et al., 2010; Ray et al., 2014). Few studies have so far explored structure-function relationships in entorhinal circuits (Schmidt-Hieber and Häusser, 2013; Domnisoru et al., 2013; Zhang et al., 2013; see Rowland and Moser, 2014 and Burgalossi and Brecht, 2014 for reviews). Thus, the functional implications of the remarkable cellular diversity of layer 2 have remained largely unresolved.

Resolving how differential spatial firing relates to principal cell types will clarify the cellular mechanisms of grid discharges and spatial input patterns to distinct subfields of the hippocampus. In the present work we aim at resolving layer 2 circuits by taking advantage of improved methodologies for identifying individual neurons recorded in freely moving animals. By cell identification and theta-locking-based classification of unidentified recordings, we provide evidence that grid and border responses are preferentially contributed by pyramidal and stellate cells, respectively.

RESULTS

To explore the cellular basis of grid cell activity in medial entorhinal cortex, we juxtacellularly recorded and labeled neurons in...
layer 2 (which contains the largest percentage of pure grid cells; Boccara et al., 2010) in awake rats trained to explore 2D environments (Tang et al., 2014). Clear grid cell discharges were rare. The clearest grid-like firing pattern in our sample of 31 identified cells (17 of which met the criteria for spatial analysis; see Experimental Procedures) was observed in the calbindin+ cell shown in Figure 1A. This neuron had pyramidal morphology, with simple dendritic arborization and a single large apical dendrite targeting a calbindin+ patch (Figure 1B; see also Ray et al., 2014). During exploratory behavior, calbindin+ neurons fired with strong theta rhythmicity and phase locked near the trough of the local field potential theta rhythm (Figure 1C; Ray et al., 2014). Spatial autocorrelation analysis of the firing pattern in the 2D environment revealed a hexagonal periodicity of firing fields (grid score = 1.07; Figure 1D), indicative of grid cell activity (Hafting et al., 2005). Because of its relatively low firing rate (~0.5 Hz) this cell was not included in the grid cell sample (see Experimental Procedures). Most other identified calbindin+ neurons had no clear spatial firing patterns.

The clearest border discharge in our sample of identified cells was observed in the calbindin+ cell shown in Figure 1E. This cell was a stellate neuron, which did not have a single apical dendrite, but instead extended multiple and widely diverging ascending dendrites; this dendritic tree spanned a vast field,
which encompassed multiple calbindin+ patches (Figure 1F; see also Ray et al., 2014). On average, spikes from calbindin− neurons were weakly modulated by the local theta rhythm (Figure 1G). In 3 out of 11 calbindin− cells from recordings with sufficient spatial coverage, we observed clear border firing patterns as in Figure 1H. While we did not observe grid cells, nonspatial firing patterns also dominated in calbindin− neurons.

While the small size of the data set of identified neurons prevented us from establishing firm structure-function relationships, four preliminary observations can be drawn: (i) grid cells are less abundant in layer 2 than previously assumed (Sargolini et al., 2006; Bocca et al., 2010; but see Mizuseki et al., 2009; Gupta et al., 2012; Bjerkes et al., 2014), and there is no one-to-one relationship between spatial discharge characteristics and cell type, (ii) calbindin+ neurons probably include grid cells, (iii) the absence of grid cells in the 22 identified calbindin− stellate neurons suggests that grid cells are rare in this cell population, and (iv) calbindin− neurons include border cells.

Currently available evidence points to a correspondence between cytochemical (calbindin+ versus calbindin−) and morphological (pyramidal versus stellate) classification of principal neurons in layer 2 (Varga et al., 2010; Kitamura et al., 2014). To further explore these relationships, we determined the percentage of calbindin+ cells in layer 2 and compared these data with related measurements in the literature (Figure S1A available online). In agreement with previous studies (Peterson et al., 1996; Kumar and Buckmaster, 2006; Varga et al., 2010), we found that layer 2 neurons consist of ~34% calbindin+ and ~53% calbindin− (and reelin+) principal cells, and ~13% interneurons (Figure S1B). We note that while Ray et al. (2014) found about 30% of calbindin+ cells, most of which were shown to have pyramidal morphology (see also Varga et al., 2010; Kitamura et al., 2014), Gatome et al. (2010) found a slightly lower fraction of putative pyramidal cells. Calbindin+ and calbindin− cells showed large quantitative differences in their morphology, but without the possible classification error in individual morphological parameters (Figures S1C and S1D). Calbindin+ cells had significantly (on average ~2.5-fold) smaller dendritic trees (Figure S1E). Dendritic trees also differed in shape between cell types. Calbindin+ cells had a single long (always apical) dendrite, which accounted on average for 63% of the total dendritic length (Figure S1E) and which was polarized toward the center of pyramidal cell patches as shown previously (Ray et al., 2014). Calbindin+ expression matched well, but not perfectly, with pyramidal morphology (Figures S1C and S1D). Calbindin− cells featured similar-length dendrites with the longest dendrite contributing on average for 33% of the total dendritic length (Figure S1E). These results are in line with published data and indicate that calbindin− and calbindin+ cells largely correspond to pyramidal and stellate neurons, respectively. However, the lack of clear morphological bimodality in layer 2 (see also Canto and Witter, 2012) implies that the correspondence between pyramidal/calbindin+ and stellate/calbindin− might not be perfect. Interestingly, the spine density in calbindin+ cells decreased as a function of distance from the soma, whereas the reverse was true for calbindin− cells (Figure S1F). These morphological differences, together with clustering of calbindin+ cells in patches and the polarization of their apical dendrites toward the center of calbindin+ patches (Ray et al., 2014), likely result in a local and overlapping sampling of inputs in neighboring calbindin+ cells, whereas neighboring calbindin− stellate cells sample large and nonoverlapping input territories.

Calbindin− stellate and calbindin+ pyramidal cells differ strongly in their temporal discharge properties (Figures 1C and 1G; Ray et al., 2014). We therefore wondered if temporal discharge properties could be used to classify layer 2 cells as putative pyramidal or stellate neurons. We used a support vector machine to classify neurons based on both the spike phase and strength of phase locking to local field potential theta oscillations, which indeed clearly segregated calbindin+ and calbindin− cells with a large distance to the separating hyperplane (Figure 2A; see Supplemental Information). To further improve the purity of assigned cells, we added a guard zone around the hyperplane separating the Gaussian kernels classifying calbindin+ (light green background) and calbindin− (gray background) cells (omitting the guard zone and classifying all cells did not qualitatively affect the results; data not shown). We tested our classifier by a bootstrapping approach (Figures S2A and S2B) and found that a large fraction of calbindin+ and calbindin− cells could be correctly assigned (Figure S2C). More importantly, the specificity of classification procedure—reflected in the purity of the resulting cell samples—was excellent, i.e., ~89% for putative calbindin− cells and ~83% for putative calbindin+ cells (Figure S2D), and even higher values for combination of identified and putatively assigned cells (Figure S2E). We further evaluated the robustness of the classifier by testing it on a larger data set of identified layer 2 neurons (Ray et al., 2014) recorded under urethane/ketamine anesthesia (Klausberger et al., 2003). We consider this a challenging test of the classifier, as theta phase and strength of locking might differ between the awake and anesthetized state.

Similarly, to the awake situation, however, the large majority of neurons recorded under anesthesia were also correctly classified (92% of calbindin+ cells, 65% of calbindin− cells, p < 0.001, bootstrap; Figure 2B, bottom), suggesting that our classification criteria work robustly and can effectively generalize across very different recording conditions (Figure 2B). Encouraged by these results, we classified the larger data set of our histologically identified layer 2 juxtacellular and tetrode recordings (classified + identified n = 193 cells).

To assess the relationship between cell identity and spatial firing properties, we pooled the nonidentified recordings, assigned to putative calbindin+ and calbindin− cells, with the recordings from histologically identified neurons. The pooled data sets included n = 99 calbindin+ and n = 94 calbindin− cells, respectively. In our first assessment of spatial discharge patterns, we attempted to classify grid and border cells solely using scores (grid score > 0.3, border score > 0.5; Solstad et al., 2008). According to visual inspection of individual rate maps, however, these criteria were not sufficiently stringent and returned a majority of weakly to nonmodulated neurons, i.e., possibly a majority of false-positive grid and border cells. To resolve this issue, we adopted the cell classification approach of Bjerkes et al. (2014), in which spatial discharge properties were only quantified in those cells that carried significant amounts of spatial information (as assessed by a spike-shuffling procedure, see Skaggs et al., 1993; Supplemental Experimental Procedures). This approach identified grid and border responses,
Neuron Layer 2 Grid and Border Cells

A. Training

B. Anaesthetized

C. Nonidentified

D. Grid score vs. Theta strength

E. put. pyramidal grid cells

F. put. stellate border cells

G. Border score vs. Theta strength

H. Identified & Theta-assigned Cb+
   (N = 99)

I. Identified & Theta-assigned Cb-
   (N = 94)

(legend on next page)
Layer 2 Grid and Border Cells

which in a majority of cases were convincing according to visual inspection. Consistent with previous studies (Hatting et al., 2005; Sargolini et al., 2006; Boccara et al., 2010; Burgalossi et al., 2011; Domnisoru et al., 2013), a fraction of layer 2 neurons (33%; n = 63 cells) were significantly spatially modulated. Weak hexagonal symmetry of spatial firing patterns was observed in both the calbindin+ and calbindin− data set, in line with previous observations (Burgalossi et al., 2011; Domnisoru et al., 2013; Schmidt-Hieber and Häusser, 2013). However, grid scores in the calbindin+ population were significantly higher than those in the calbindin− population (p = 0.000046, Mann-Whitney U test; Figures 2D and 2E), consistent with observations from the identified data set (Figure 1). On the other hand, in line with observations from the identified data set (Figure 1), calbindin− cells had significantly higher border scores than calbindin+ cells (Figure 2G; p = 0.0012, Mann-Whitney U test). Border discharges in calbindin+ cells are shown in Figure 2F, which also includes an example where border firing was confirmed by a border test (Solstad et al., 2008; Lever et al., 2009). Thus, according to the grid and border scores shown in Figures 2D and 2G, putative pyramidal and stellate cells have significantly different, but overlapping, spatial properties.

Figure 2 gives an overview of the spatial response properties of our pooled calbindin+ and calbindin− data sets, respectively (see also Figure S3). The majority of both calbindin+ and calbindin− neurons showed no significant spatial selectivity. Grid patterns were significantly more common in the calbindin+ population, where 19% (19/99) of the cells passed our grid cell criteria, compared to only 3% (3/94) in the calbindin− population (p = 0.00046, Fisher’s exact test). A higher fraction of calbindin− cells passed the border cell criterion (11% calbindin−, 10/94 cells; versus 1% calbindin+, 1/99 cells), and this difference was statistically significant (p = 0.0042, Fisher’s exact test). These data confirm and extend the conclusion from our recordings of identified cells and indicate that grid cells are preferentially recruited from the calbindin+ population, while border responses preferentially occur in calbindin− cells.

Unlike many studies based on tetrode recordings (Sargolini et al., 2006; Boccara et al., 2010; but see Zhang et al., 2013), a substantial fraction of cells showed head-direction selectivity both in identified and theta-assigned calbindin+ and calbindin− cells (Figure S4). Head-direction selectivity was more common in calbindin+ (19%, 19 out of 99 cells) than in calbindin− cells (12%, 11 out of 94 cells), but this difference was not significant (p = 0.17, Fisher’s exact test), and both classes contained pure as well as conjunctive responses (Sargolini et al., 2006).

The grid and border cells recorded here showed systematic differences in spike locking to local field potential theta oscillations (Figure 3A). Spikes from most grid cells were strongly entrained by the theta rhythm, with strong phase locking (Figure 3B) and a phase preference near the theta trough (Figure 3C; p = 0.000000027, Rayleigh’s test for nonuniformity). The modulation of spiking activity of border cells by the theta rhythm was significantly weaker than in grid cells (Figure 3B; p = 0.0013, Mann-Whitney U test) and showed on average only a weak, nonsignificant phase preference for the theta
peak (Figure 3C; \(p = 0.21\), Rayleigh’s test for nonuniformity), which differed significantly from the phase preference of grid cells (Figures 3B and 3C, \(p = 0.000008\), parametric Watson-Williams multisample test). Thus, in layer 2 grid and border signals mirrored the temporal differences between calbindin\(^+\) pyramidal and calbindin\(^-\) stellate cells reported earlier (Ray et al., 2014).

**DISCUSSION**

Relating functionally defined discharge patterns to principal cell diversity is an unresolved issue in cortical physiology. In layer 2 of medial entorhinal cortex, most studies suggested that spatially modulated responses are common, and that grid firing patterns are contributed by both stellate and pyramidal neurons (Burgalessi et al., 2011; Schmidt-Hieber and Häusser, 2013; Domnisoru et al., 2013; Zhang et al., 2013). In line with such evidence, we observed a consistent fraction of spatially modulated neurons in layer 2, and weakly hexagonal firing patterns in both stellate and pyramidal neurons. At the same time, however, most grid patterns that met our grid score and spatial information criteria (see **Supplemental Experimental Procedures**) were classified as putative calbindin\(^+\) pyramidal cells (see Figure S3A). Border responses, on the other hand, were predominantly observed in the calbindin\(^-\) stellate population (Figure S3B). Our data indicate a strong interdependence between cell type and spatial discharge pattern in layer 2, where a calbindin\(^+\) cell is about six times more likely to be a grid cell and ten times less likely to be a border cell than a calbindin\(^-\) neuron. Our confidence in classification is based on the striking differences between calbindin\(^+\) and calbindin\(^-\) cells in their temporal discharge properties (Ray et al., 2014), the assessment of classification quality by our bootstrapping approach, and the robustness of classification across widely differing recording conditions. It is important, however, to note that our conclusions rest on the validity and accuracy of our classification procedure.

A key finding from our work is that layer 2 principal cells can be classified with high accuracy by their distinct temporal discharge properties. Such classification can be extended to a large number of unidentified layer 2 recordings from other laboratories, provided that the required histology and local field potential data have been collected. To this end we provide our classification training data set (**Table S1**) and a custom-written MATLAB function (**Supplemental Information**, **Note S1**). Such post hoc assignment of principal cell types to recordings—i.e., supplying identity to formerly blind extracellular recordings—could be instrumental for understanding principal cell diversity and cortical microcircuitry.

Calbindin\(^+\) pyramidal cells might be predetermined for grid cell function as they receive cholinergic inputs, are strongly theta modulated, and are arranged in a hexagonal grid (Ray et al., 2014). We suggested an “isomorphic mapping hypothesis,” according to which an anatomical grid of pyramidal cells (Ray et al., 2014) generates grid cell activity (Brecht et al., 2014) and is an embodiment of the brain’s representation of space in hexagonal grids. Representing grid discharge by a “cortical grid” might offer similar advantages as isomorphic representations of body parts, as barrel fields (Woolsey and Van der Loos, 1970), or nose stripes (Catania et al., 1993), in somatosensory cortices of tactile specialists. Notably, the local similarity of grid cell discharges is high, as neighboring grid cells share the same grid orientation and scaling and are phase coupled even across distinct environments (Hafting et al., 2005; Fyhn et al., 2007). We speculate that calbindin\(^+\) pyramidal neuron clustering and apical dendrite bundling in patches (Ray et al., 2014) might impose this local similarity of grid discharges. A surprising implication of our data is that the spatial input to the dentate gyrus is provided mainly by stellate border cells, whereas pyramidal grid cells do not feed into this pathway (Kitamura et al., 2014; Ray et al., 2014). Border responses arise in stellate neurons, with long and widely diverging dendritic trees, i.e., such discharge patterns may result from a relatively global sampling of incoming inputs in medial entorhinal cortex and help generate place cell activity (Bjørknes et al., 2014; Bush et al., 2014). Recognizing the functional dichotomy of pyramidal and stellate cells in layer 2 will help elucidate how spatial discharge patterns arise in cortical microcircuits.

**EXPERIMENTAL PROCEDURES**

All experimental procedures were performed according to the German guidelines on animal welfare under the supervision of local ethics committees. Juxtacellular recordings and tetrode recordings in freely moving animals were obtained in male Wistar and Long-Evans rats (150–250 g), which were habituated to the behavioral arena and trained for 3–7 days. Experimental procedures were performed as previously described (Burgalessi et al., 2011; Herfst et al., 2012) with the exception that methodological developments allowed us to identify neurons in drug-free animals (Tang et al., 2014; see also **Supplemental Experimental Procedures**). Some of the data have been published in a previous report (Ray et al., 2014). Recordings in anesthetized animals were performed under urethane/ketamine/xylazine (Klausberger et al., 2003). Juxtacellularly labeled neurons were visualized with streptavidin conjugated to Alexa 546 (1:1,000). A Hilbert transform was used for assigning instantaneous theta phase of each spike based on theta in the local field potential in the spike-theta phase analysis. The spatial periodicity of recorded neurons was assessed by spatial autocorrelations. Grid scores were calculated as previously described (Barry et al., 2012) by taking a circular sample of the spatial autocorrelogram, centered on, but excluding the central peak. To determine the modulation of a cell firing along a border, we determined border scores as previously described or performed border tests (Solstad et al., 2008; Lever et al., 2009). Head-direction tuning was measured as the eccentricity of the circular distribution of firing rates. Classification based on strength of locking to theta phase (S) and preferred theta phase angle (\(\phi\)) was done by building a support vector machine, trained on the vectors (cos(\(\phi\)) - S, sin(\(\phi\)) - S) using a Gaussian radial basis function kernel. Classification of nonidentified cells into putative calbindin\(^+\) and calbindin\(^-\) cells was performed by applying a conservative classification threshold, where we did not classify cells close to the separating hyperplane. Detailed experimental and analytical procedures are provided in the **Supplemental Information**.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2014.11.009.

**AUTHOR CONTRIBUTIONS**

Q.T. and A.B. performed juxtacellular recordings. C.L.E. and Q.T. performed tetrode recordings. S.R., R.N., and H.S. performed and analyzed anatomical experiments. C.L.E. and D.S. developed the classifier and C.L.E. and Q.T. analyzed electrophysiology data. A.B. and M.B. conceived of the project and supervised experiments. All authors contributed to the writing of the manuscript.
ACKNOWLEDGMENTS

This work was supported by Humboldt-Universität zu Berlin, BCCN Berlin (German Federal Ministry of Education and Research BMBF, Förderkennzeichen 01GG0010A), NeuroCure, the Neuro-Behavior ERC grant, and the Gottfried Wilhelm Leibniz Prize of the DFG. We thank Moritz von Heimendahl for programming, and Andreae Neukirchner, Juliane Steger, John Tukker, and Undine Schnieweß. We are thankful to Francesca Sargolini, May-Britt and Edvard Mose, and the other authors (Sargolini et al., 2009), who generously provided access to grid cell data, which were helpful in assembling an earlier version of this manuscript.

Accepted: November 6, 2014
Published: December 4, 2014

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