Title:

cfos principal cells and interneurons are strongly reactivated by sharp wave ripples

Authors:

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Abstract

The hippocampal formation is central for the learning and consolidation of spatial memories. While it is known that the high-frequency oscillations, called sharp wave ripples, play a critical role for memory processes, it is unclear if they interact with the memory engram and spatial engram cells. Here we identify the effect of these oscillations on engram cells as mice explored two environments over several days. We found that both principal cells and interneurons are part of the cfos-tagged engram. cfos-tagged principal cells, place cells and interneurons are highly reactivated by SWRs, whereas none of the negatively SWR-modulated cells are part of the engram. Together, our findings reveal a critical link between cellular and network mechanisms for memory formation and imply that interneurons play a key role in it.

Main Text

How experiences and memories are encoded in the brain remains a central question in neurosciences. At the cellular level, a subset of neurons, called engram cells, which are co-active during an experience are central for encoding and storing the memory of that experience. In the hippocampus, cfos expressing engram cells are required for memory recall, their deletion interferes with memory and their reactivation triggers memory recall (Liu *et al.*, 2012; Tayler *et al.*, 2013; Tanaka *et al.*, 2014; Ryan *et al.*, 2015; Josselyn & Tonegawa, 2020). A key structure for all types of learning and memory, the hippocampus, is also a central region for encoding spatial environments and for navigation. Hippocampal place cells provide real-time information on our location in an environment (O'Keefe & Dostrovsky, 1971) and they support spatial navigation (Moser *et al.*, 2017). Even though these spatial cells are often part of an engram (Trouche *et al.*, 2016; Tanaka *et al.*, 2018; Pettit *et al.*, 2022), their role in the engram is debated and the circuit mechanisms regulating their recruitment to the engram remain unknown.

At the network level, fast oscillating brain waves (150 - 250 Hz) in the hippocampus called sharp wave ripples (SWRs) are known (i) to reactivate neuronal sequences during offline periods (Wilson & McNaughton, 1994; Skaggs & McNaughton, 1996; Lee & Wilson, 2002; Foster & Wilson, 2006; O'Neill *et al.*, 2008) and (ii) to be critical for the consolidation of memories post-learning (Girardeau *et al.*, 2009; Ego-Stengel & Wilson, 2010; Van De Ven *et al.*, 2016; Michon *et al.*, 2019). Despite being crucial for memory, the relationship of SWRs with engram cells remains unclear. It is widely assumed that these fast network oscillations reactivate neurons that previously participated in an experience (Wilson & McNaughton, 1994; Joo & Frank, 2018; De La Prida, 2020), and that repeated co-activation induces plasticity mechanisms (Dupret *et al.*, 2010; Sadowski *et al.*, 2016; Norimoto *et al.*, 2018) and strengthens the relation to the experience (Maboudi *et al.*, 2024; Yang *et al.*, 2024).

Hippocampal interneurons are increasingly thought of as central in orchestrating oscillations and synchronous activity (Stark *et al.*, 2014; Allen & Monyer, 2015; Gan *et al.*, 2017). They are also involved in SWR generation and play a regulating role for SWRs likely through specific activity patterns before and during SWRs (Valero *et al.*, 2015; Joo & Frank, 2018; Vancura *et al.*, 2023). Yet, it is unknown if the role of interneurons goes beyond timing the oscillations e.g. if they become part of the hippocampal engram. Moreover, the *in vivo* activity of cfos expressing engram neurons during SWRs, and therefore the link between the cellular and circuit mechanisms for memory encoding and consolidation, is to date not well established.

Spatial encoding induces cfos expression in both principal cells and interneurons of the CA1 area of the hippocampus

To improve our understanding of how spatial encoding of environments is linked to the activity of engram neurons and brain oscillations, we used *cfos-tTA* (tetracycline-transactivator) mice (Garner *et al*., 2012) and opto-tagging of cfos expressing cells using an AAV-TRE-ChR2-mKate2 viral vector (**Fig. 1A**). Doxycycline was removed from the diet (DOX OFF) allowing expression of ChR2 and mKate2 in cfos-expressing cells. 40-46 hours after DOX removal mice were exposed to two novel environments (Context A and Context B) and then returned to the first environment (Context A'). After this exposure, DOX-containing food was provided again, to close the tagging window (**Fig. 1B**). In the subsequent six to ten days the *in vivo* activity of extracellular units and the local field potential (LFP) were recorded in the dorsal CA1 (**fig. S1A**), while the mice were reintroduced to the two environments (Context A, B, A'), as well as during the pre, and post-behavior rest-sessions on each day (R1 and R2 respectively; **Fig. 1B**). Post-hoc identification of cfos-tagged mKate-expressing cells, together with electrode tracks (**Fig. 1C, fig. S2**) were used to confirm the injection and recording site respectively. Optotagged, cfos-expressing cells were identified at the end of every *in vivo* recording, as units that fired with five SD above baseline during optogenetic stimulation, tested with 100 x 15 ms pulses at 473 nm and using a 561nm laser as control (**Fig. 1D, fig. S1B**). Based on optotagging across 7 mice and 513 total cells, 70 cfos expressing cells were identified. Within the cfos-tagged group of cells, 81.4% of the cells were identified as putative principal cells (**Fig. 1E, F**) and 18.6% as putative interneurons (**Fig. 1E, G, fig. S2)**, revealing that putative interneurons are part of the engram. Similarly, among the non-cfos-tagged (untagged) population, 87.4% of the cells were identified as putative principal cells and 12.6% as putative interneurons (**Fig. 1E**).

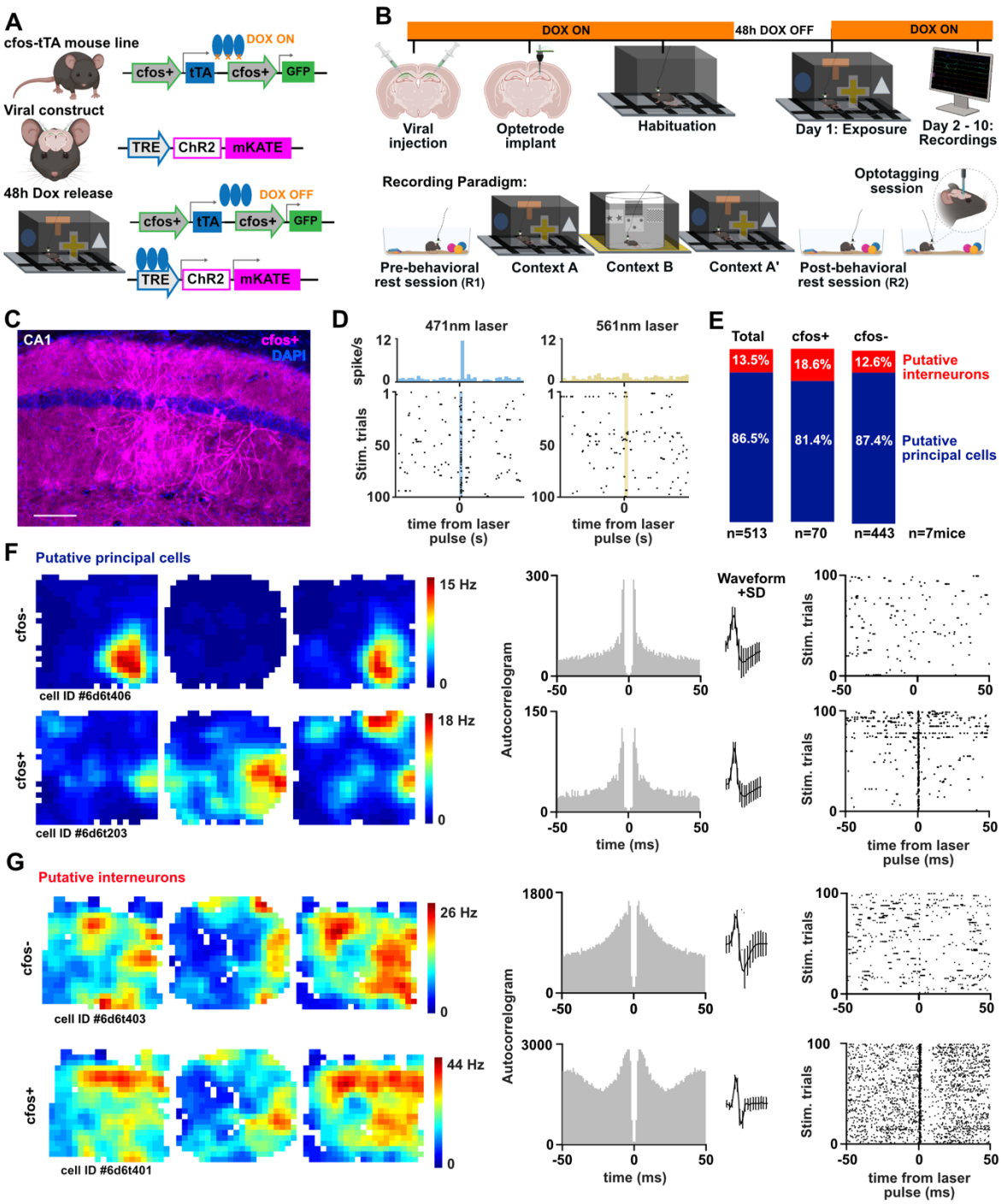

Fig. 1. During spatial foraging cfos is expressed both in principal cells and interneurons of the CA1 area of the hippocampus.
(**A**) cfos-tTA mouse line injected with AAV-TRE-ChR2-mKate2, allows for temporally restricted tagging of cfos-expressing neurons. (**B**) Experimental paradigm, with tagging window during the exposure to the new environments and the recording paradigm during revisiting these environments. (**C**) Overview of cells tagged *in vivo* in the hippocampal CA1 area. (Scale bar: 100µm) (**D**) Optotagging, with a strong response to ChR2 activation (using 471nm laser), was used to identify cfos-tagged cells within the recorded units. (**E**) Classification of cell types showed that cfos-tagged cells are a composite of both principal neurons and interneurons (p=0.1477, Binomial test) (**F**) Examples of the spatial maps in context A, B and A', the autocorrelogram, waveform and response to optogenetic stimulation of cfos- and cfos+ putative principal cells and (**G**) As in (F) but for cfos- and cfos+ putative interneurons.

cfos-tagged principal cells are highly reactivated by SWRs

Out of the 444 principal cells, 13% were identified as cfos-tagged and the remaining 87% as untagged cells. 79% of the cfos-tagged putative principal cells were place cells, whereas 73% of the untagged putative principal cells were place cells (**Fig. 2A**). Examining the properties of place cells, we found a similar average firing between cfos-tagged and untagged place cells, but a higher peak firing rate (p=0.0279) and burst index (p=0.0466, Mann Whitney or MW test; see **Supplementary Table 1** for more details on the statistics performed) in cfos-tagged place cells (**Fig. 2B;** properties of principal and principal non-place cells are displayed in the **fig. S3**). cfos-tagged place cells showed no difference in their spatial peak firing rates but had larger place fields (p=0.0010) with lower information content (bits/spike) (p=0.0142, MW test; **Fig. 2C**; Context A), in agreement with earlier findings (Tanaka *et al.*, 2018), whereas the number of place fields was equal (**fig. S3C**). Based on the place maps in the different environments (**Fig. 2D**) we found that the stability (Context A versus revisit A') and the remapping (Context A versus Context B) in our task were equal between cfos-tagged and untagged place cells (**Fig. 2E**). Since spike patterns in the theta range have been shown to be important for correct memory encoding (Viana Da Silva *et al.*, 2024) and cfos-tagged cells where previously shown to be theta burst modulated during encoding (Tanaka *et al.*, 2018), we wanted to examine place cell activity in relation to theta after the encoding phase. The spike oscillation frequencies of CA1 place cells within the theta band were similarly distributed for cfos-tagged and untagged cells (**Fig. 2F**). We further determined the theta modulation index and found that untagged and cfos-tagged place cells were equally modulated by theta in our paradigm (**Fig. 2G-H**).

To determine whether cfos-tagged cells are preferentially reactivated by SWRs, we calculated the SWR modulation index as the activity of neurons during SWRs relative to the 100 ms prior to the SWR (**Fig. 2I-J**, see *Methods*). cfos-tagged cells showed higher average modulation by SWRs than untagged cells, both during the pre- and post-behavior rest-sessions (R2: **Fig. 2K,** p=0.0040**;** R1: **fig. S3H,** p=0.0015, MW test). The percentage of strongly modulated place cells was larger in the cfos-tagged group (R1: 88.89% vs 59.07%, p<0,0001; R2 88.89% vs 68.68%, p=0,0021, Binomial test) (**Fig. 2K, Supplementary Table 2-4**).

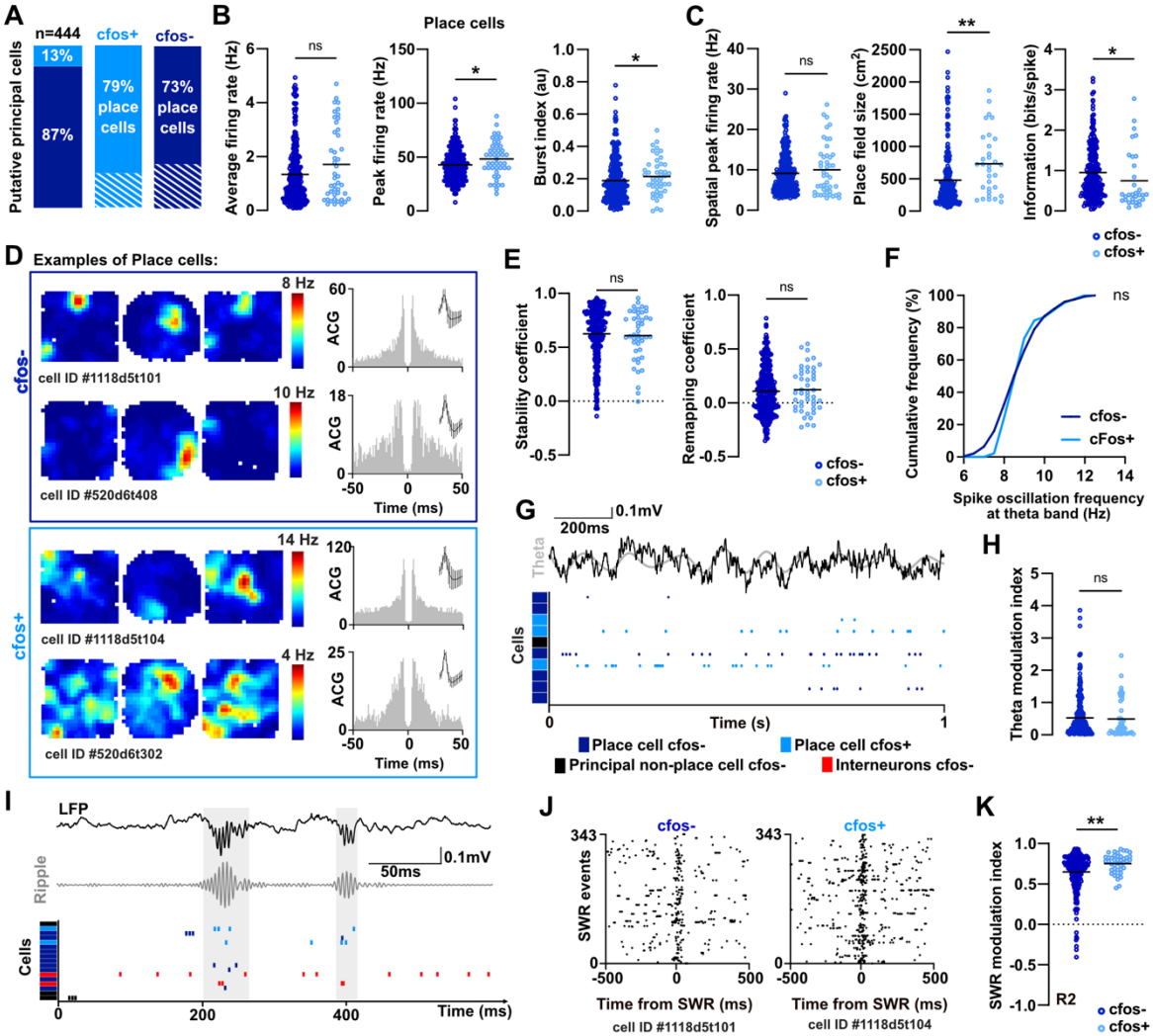

Fig. 2 cfos-tagged principal cells are preferentially reactivated by SWRs.
(**A**) Ratio of cfos-tagging within principal cells, and ratios of place cells within cfos-tagged and untagged cells (p=0.372, binomial test). (**B**) cfos-tagged place cells have same average firing rates but increased peak firing rates and burst index. (**C**) The spatial peak firing rate is equal (p=0.8720), whereas the place field size is larger (p=0.0010) and the information score is lower (p=0.0142, MW test) for cfos-tagged cells (in Context A). (**D**) Examples of place cells in sequential recordings in Context A, B and revisiting A'. (**E**) Stability (p=0.4830) and remapping (p=0.6169) coefficients of cfos-tagged place cells are unaltered. (**F**) The spike oscillation frequency of cfos-tagged and untagged place cells is equally distributed (p=0.2026, KS test). (**G**) Example of raw and theta filtered LFP signal with associated unit activity. (**H**) Theta modulation index is equal between cfos-tagged place cells and untagged place cells (p=0.1419, MW test). (**I**) Example of unit activity during SWRs. (**J**) Activity of individual (cfos+ and cfos-) place cells, during all detected SWRs within the same recording session, across a time window of 1s (SWRs centered at 0 ms). (**K**) cfos-tagged principal cells are stronger modulated by SWRs than untagged cells during R2 (p=0.0040, MW test). In **B**, **C**, **E**, **H**, **K** circles correspond to recorded place cells, line to the mean. *ns: P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001.*

cfos-tagged interneurons display stronger reactivation by SWRs

Since we identified that a fraction of the cfos-tagged cells were interneurons, which we confirmed based on their firing rate, autocorrelogram and spike features (**Fig. 1, 3A and fig. S4**), and since the idea of an interneuron contribution to the hippocampal engram has received

124 little attention to date, we found it important to characterize tagged engram interneurons. Out
125 of the 69 putative interneurons, 18.8% were identified as cfos-tagged and the remaining 81.2%
126 as untagged non-cfos cells (**Fig. 3B**), which is similar to the ratio of cfos-tagged cells among
127 principal cells (13% cfos-tagged vs 87% untagged, see **Fig. 2A**). Although the average firing
128 rates and peak firing rates of cfos and non-cfos interneurons were similar, we found that cfos-
129 tagged cells have a significantly higher speed modulation (p=0.0426, MW test, **Fig. 3C;** see
130 **Supplementary Table 5** for more details on the statistics performed). It is also noteworthy
131 that none of the cfos-tagged cells showed a negative speed score, whereas 36% of the
132 untagged cells have a negative speed score (based on speeds greater than 2cm/s) (**Fig. 3C**).
133 Since the spiking of interneurons is also modulated by theta oscillations (**Fig. 3D**) we further
134 characterized if there are differences in the theta modulation properties. Even though we found
135 no difference in the theta modulation of spikes of cfos-tagged interneurons (**fig. S4D**), the
136 fraction of untagged interneurons, which show no significant spike oscillation frequency within
137 the theta band is absent in the cfos-tagged population (**Fig. 3E**). Since it is known that
138 interneurons play a critical role for SWRs (Stark *et al.*, 2014; Buzsáki, 2015; Gan *et al.*, 2017)
139 and we found cfos-tagged interneurons, we therefore characterized the modulation of putative
140 interneurons by SWRs (**Fig. 3F**) and found that cfos-tagged interneurons are stronger SWR-
141 modulated compared to untagged neurons (p=0.0004 for R2: **Fig. 3G-H;** p=0.0143 for R1,
142 MW test **fig. S4F**). Also, the percentage of strongly modulated (SWR modulation index above
143 0.5) interneurons was larger in the cfos-tagged group in R2 (p<0,0001; 100% vs 48.21%,
144 Binomial test). None of the cfos-tagged interneurons showed a negative SWR-modulation,
145 whereas 28.57% of the untagged interneurons were negatively SWR-modulated in R2
146 (p=0,026, Binomial test; **Fig. 3H, Supplementary Tables 2-4**).
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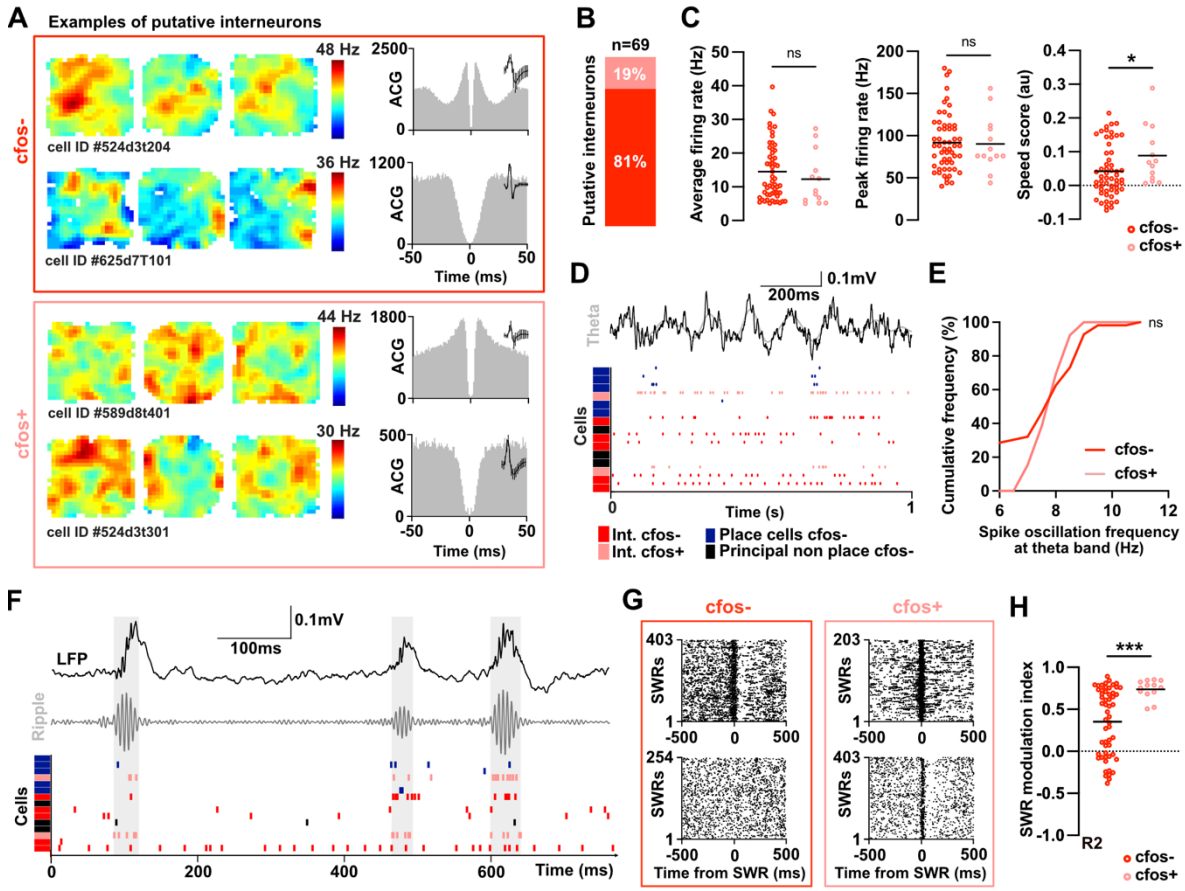

Fig. 3. cfos-tagged interneurons are strongly reactivated by SWRs.
(**A**) Examples of the place maps and autocorrelograms (ACG) of putative interneurons, untagged (cfos-) and cfos-tagged (cfos+). (**B**) Ratio of cfos-tagged cells within putative interneurons. (**C**) cfos-tagged interneurons have the same average firing rates (p=0.2761) and peak firing rates (p=0.8763) as untagged interneurons, whereas they have a higher speed score (p=0.0426, MW test). (**D**) Example of raw (black) and theta filtered (gray) LFP signal and simultaneous unit activity. (**E**) While the spike oscillation frequency of cfos-tagged and untagged putative interneurons is not differently distributed (p=0.2852, KS test), the fraction of interneurons with no apparent spike oscillation frequency within the theta band is absent from the cfos-tagged interneurons. (**F**) Examples of unit activity during SWR-oscillations. (**G**) Activity of individual (cfos+ and cfos-) putative interneurons, during all detected SWRs within the recording session, across time windows of 1s (SWRs centered at 0 ms). (**H**) cfos-tagged putative interneurons are stronger modulated by SWRs than untagged cells during R2 (p=0.0004, MW test). *ns: P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001.*

Characteristics of correlated activity of SWRs and cfos-tagged cells

To better understand the correlated activity of SWRs and cfos-tagged cells, we wanted to further clarify if SWR properties play a role in the likelihood to re-activate cfos-tagged cells and/or if there are differences in the timed activity of cfos-tagged cells during SWRs. To first improve our understanding if different SWRs (e.g., larger or longer) are more likely to reactivate cfos-tagged cells, we tested for the amplitude and duration of the SWRs that re-activated at least 1 or more cfos-tagged cells in our recordings. Even though there was no difference in the strength of the SWRs that reactivated cfos-tagged cells (**Fig. 4A;** see **Supplementary Table 6** for more details on the statistics performed), the duration of the

158 SWRs that reactivated cfos-tagged cells was longer ($p<0.0001$, MW test; **Fig. 4B**). To test for
159 the possibility that changes across days (e.g. as the animals become increasingly familiar with
160 the environment) would contribute to this effect, we first tested for the duration of the SWRs
161 across days. We found that in R2, the SWR duration remained similar over the recording days
162 (**fig. S5D**). Whereas the modulation index of cfos-tagged showed an increase over the
163 recording days for principal cells ($p=0.0416$) and interneurons ($p=0.0105$, simple linear
164 regression, **fig. S5C-E**).
165 Since it is known that different neuron types display differences in the preferential firing during
166 SWRs (Klausberger & Somogyi, 2008; Dudok *et al.*, 2021; Topolnik & Tamboli, 2022), we
167 characterized in detail the re-activation profile of cfos-tagged neurons during SWRs. First, we
168 calculated the average population activity profile of cfos-tagged and untagged cells, both for
169 principal cells and interneurons. We observed that cfos-tagged principal cells and interneurons
170 have a much larger probability of spiking during the SWR duration (0-1), compared to non-
171 tagged neurons ($p<0.0001$, Kolmogorov-Smirnov test; R2: **Fig. 4C**). Which is also apparent
172 when separating into place and non-place cells and during R1 (**fig. S6**). To determine whether
173 cfos-tagged neurons fire at particular times during SWRs, we further defined the timing of the
174 peak rate as happening early, at the center or late in the ripple (**Fig. 4D**). cfos-tagged cells
175 show a shift towards the center and later epochs of the SWR ($p=0.0472$ for principal neurons
176 and $p=0.0042$ for interneurons, Chi-square test; **Fig. 4E-F**). Of note, in cfos-tagged
177 interneurons we found an absence of the units in the epochs of pre-SWR, early and post-
178 SWR.

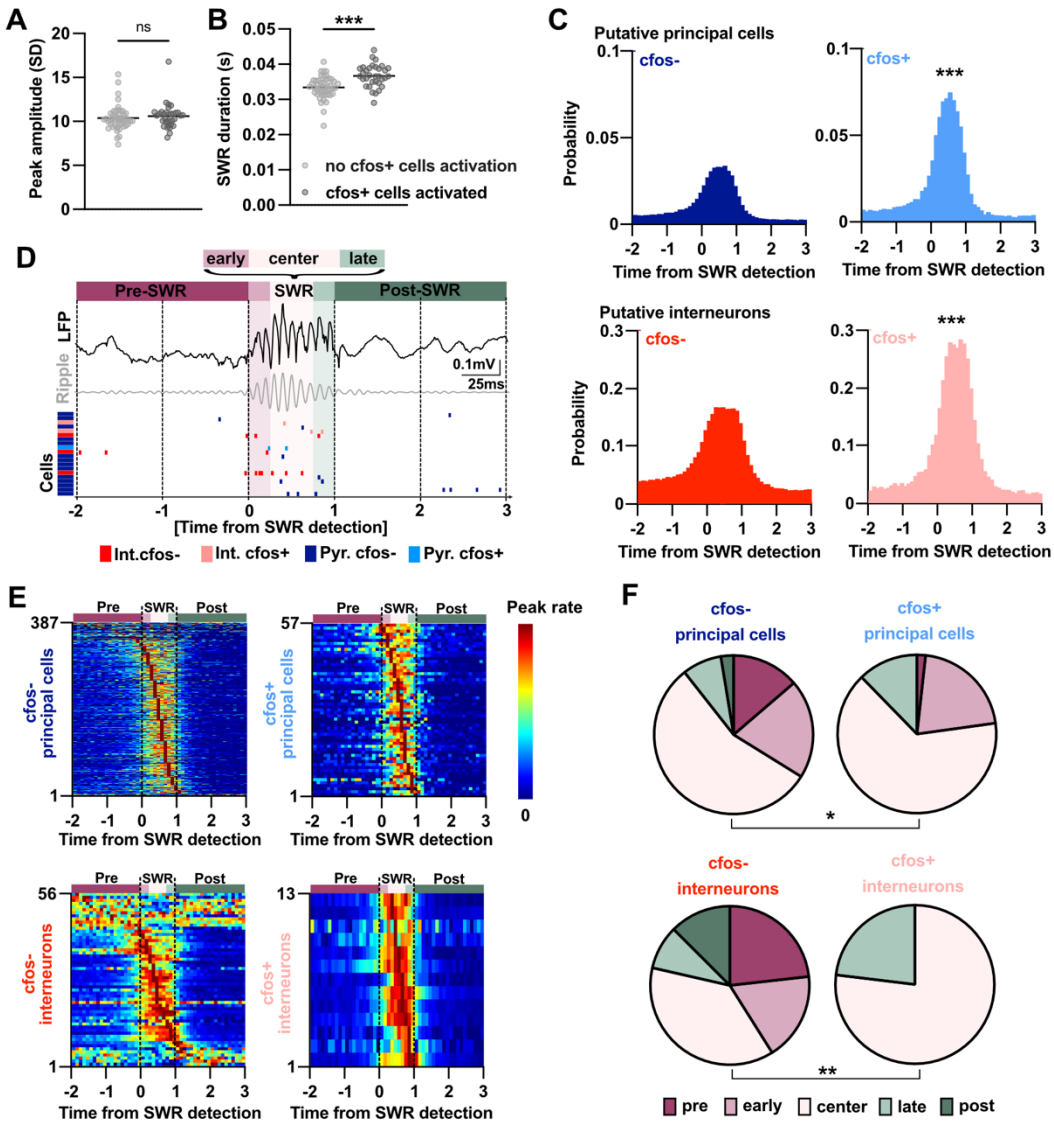

Fig. 4. Characteristics of correlated activity of SWRs and cfos-tagged cells
(**A**) Peak amplitude of SWRs (in R2) activating only untagged (cfos-) or at least one cfos-tagged (cfos+) neuron (p=0.2603, MW test). (**B**) Duration of SWRs activating only untagged (cfos-) or at least one cfos-tagged (cfos+) neuron (p<0.0001, MW test). (**C**) SWR-associated population activity (in R2) for cfos-tagged (cfos+) and untagged putative principal cells (upper panels) and interneurons (lower panels) (p<0.0001 for all comparisons) (**D**) Example of SWR-associated spike timings with early, center and late epochs depicted. (**E**) SWR activity of the individual units sorted by the relative time of their peak firing rate for principal cells and interneurons. (**F**) Units grouped by their peak firing into pre-SWR, during SWR (early, center, late) and post-SWR, revealed fewer cfos-tagged neurons with peak activity during pre-SWR and post-SWR compared to untagged principal units (p=0.0472, , upper panels) and an absence of preferentially pre- and early firing units in the cfos-tagged (cfos+) putative interneurons (p=0.0042, Chi^2 test; lower panel). *ns: P > 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001.*

Discussion

In this study, we characterized the interaction between three key components of spatial memories: cfos-expressing neurons, sharp wave ripples, and place cells, using *in vivo* single unit recordings and cfos-tagging in the hippocampal CA1 area. While most of the spatial properties of place cells were maintained between cfos-tagged neurons and untagged

neurons, we found cfos-tagged neurons had a higher burst firing rate and a lower information score per spike. While a recent study suggested that Fos expression is more likely to occur in hippocampal place cells and that Fos-expressing place cells encode reliable maps of the space (Pettit *et al.*, 2022), this is contradictory to the previously found low stability of place fields after 12 hours (Tanaka *et al.*, 2018). Two major differences between our study and Tanaka and colleagues can explain that we found no difference in the stability and remapping: (i) our engram reflects the exposure to both environments (context A and B) as we wanted to understand whether spatial remapping was maintained when the experience was composed by more than one context, encoded in a single engram (Cai *et al.*, 2016) (ii) in our paradigm less than one hour separates the exposure to context A and A' (in contrast to the 12h tested). At the same time, our data indicates no strong preference for cfos-tagging of place cells (with 79% of cfos-tagged and 73% of untagged cells cells being place cells). Our data therefore supports the notion that even though a large fraction of CA1 cfos cells are spatial, they may also encode more than a spatial map of an environment as suggested previously (Tanaka *et al.*, 2018).

Importantly, we observed that the modulation by SWRs of principal and place cells was stronger for cfos-tagged than for untagged non-cfos place cells. This is also in line with two recent reports that found that an elevated activity of neurons during quiet rest is a predictor of future IEG-tagging (Monasterio *et al.*, 2024) and that co-reactivation of neuronal ensembles during burst activities links memories across days (Zaki *et al.*, 2024). While these activity patterns were not identified as SWRs (Monasterio *et al.*, 2024; Zaki *et al.*, 2024), we now found that the SWRs at the encoding and re-exposure highly modulate cfos-tagged cells and that none of the negatively or weakly SWR-modulated principal cells were part of the cfos-tagged population. We further noted that as the mice explored the environments daily, the SWR modulation of the initially cfos-tagged cells increased over days.

Strikingly, we identify that also putative interneurons are part of the cfos-tagged cells and that the tagged interneurons are also highly modulated by SWRs. Conversely, we found that interneurons which show a negative modulation by SWRs, but also interneurons with a negative speed modulation and interneurons that are not intrinsically modulated by theta were always untagged. One potential explanation would be that these interneurons are Sncg+ / cholecystokinin-expressing (CCK+) basket cells, which have been described to be quiet during running activity, and thus during high theta activity, and are active during quiet times / rest but silent during SWRs (Klausberger *et al.*, 2005; Dudok *et al.*, 2021). This is in line with a recent finding that this interneuron type is part of an activity burst, termed BARRs, that is acting antagonistic to the SWRs (Karaba *et al.*, 2024). Taken together, given the spatial nature of the task our findings could indicate an important role of interneurons for encoding the environment and to perform spatial navigation within it, which aligns with the recent finding

that the overlap ensemble of an aversive memory with a neutral was enriched in inhibitory neurons (Zaki *et al.*, 2024). Our findings also have implications for the understanding of Alzheimer's Disease, where newly occurring memory engrams have been found to interfere with memory (Poll *et al.*, 2020), which may be linked to deficits in inhibitory connections and SWRs (Prince *et al.*, 2021).

Overall, our data provide strong support of the hypothesis that SWRs play a key role in reactivating the engram cells encoding an environment and therefore provide evidence for a crucial link between these two encoding mechanisms at the cellular and network level.

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