Hippocampus does not appear to be a major target of thalamic nucleus reuniens

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Abstract

The prefrontal – hippocampal – entorhinal system is perhaps the most widely-studied circuit in cognitive and systems neuroscience, due to its role in supporting cognitive functions such as working memory and decision-making. Disrupted communication within this circuit is a key feature of disorders such as schizophrenia and dementia. Nucleus reuniens (NRe) is a midline thalamic nucleus that sits at the nexus of this circuit, linking these regions together. As there are no direct projections from prefrontal cortex to hippocampus, the accepted model is that the NRe mediates prefrontal drive of hippocampal activity, although these connections are poorly defined at the cellular and synaptic level. Using *ex vivo* optogenetics and electrophysiology, alongside monosynaptic circuit-tracing, we sought to test the mechanisms through which NRe could drive hippocampal activity. Unexpectedly, we found no evidence that pyramidal cells in CA1 receive input from NRe, with midline thalamic input to hippocampus proper appearing selective for GABAergic interneurons. In other regions targeted by NRe, we found that pyramidal cells in prosubiculum and subiculum received synaptic inputs from NRe that were at least an order of magnitude weaker than those in prefrontal or entorhinal cortices. We conclude that, contrary to widely-held assumptions in the field, the hippocampus is not a major target of nucleus reuniens.

Introduction

Numerous aspects of cognition, such as memory [1] and decision-making [2], require the communication of information between prefrontal cortex (PFC), hippocampus (HPC), and entorhinal cortex (EC). Thalamic nucleus reuniens (NRe) sits at the nexus of this circuit and is implicated in conditions such a schizophrenia and epilepsy [3]. This circuit includes both direct connections between the cortical and hippocampal regions, as well as indirect routes via thalamus, making interpretation of the function of any individual component particularly challenging. Synchronous oscillations occur between PFC and HPC at theta frequency

during decision-making [4,5], between EC and PFC at theta frequency during associative learning [6], and between EC and HPC at theta and gamma frequencies during spatial learning [7]. There is, however, an *anatomical anomaly* within this circuitry: despite the functional importance of harmonised activity, PFC does not project directly to HPC [see 8 for our recent confirmation]. The accepted view of this circuit is that NRe directly mediates communication between PFC and HPC [e.g. 9,10], which implicates NRe in cognitive functions such as working memory [11,12], and leading many to believe that the main function of NRe is to mediate prefrontal control over the hippocampus. However, NRe inactivation or lesion studies often fail to provide strong evidence for a role in spatial memory acquisition [13].

Anatomically, NRe forms reciprocal connections with PFC and subiculum, while uniquely for the thalamus, it also sends direct efferents to CA1; these efferents terminate alongside EC axons in stratum lacunosum-moleculare (SL-M) [14,15]. Hippocampal return projections to NRe arise entirely from subiculum, with neither dorsal nor ventral CA1 forming monosynaptic connections with neurons in NRe [16]. NRe sends projections to EC [17], but these are effectively not reciprocated [18] with perhaps only 2% of superficial EC neurons projecting to NRe [16]. Functionally, although electrical stimulation in NRe fails to elicit spiking in the CA1 pyramidal layer *in vivo* [19], it has been widely assumed that NRe targets CA1 pyramidal cells [11]. This assumption is reasonable as we are unaware of any cortical region in which thalamic inputs do *not* target pyramidal cells. Surprisingly, despite NRe's important role in goal-directed spatial navigation [20] and working memory [21], NRe projections to CA1 have remained poorly defined. We previously reported that both NRe and entorhinal fibres terminate in stratum lacunosum-moleculare (SL-M) of CA1 where they target neurogliaform cells [22]. Optogenetic stimulation of NRe inputs to these neurogliaform cells elicits monosynaptic EPSCs that are defined by large NMDA receptor-mediated components.

Here, we sought to determine the nature of NRe inputs onto CA1 pyramidal cells. We hypothesised that a similarly large NMDA-R component in pyramidal cells to what we

observed in neurogliaform cells could underlie observation that NRe-fEPSPs are greatly increased in CA1 during EC input coactivation [23]. However, we found no evidence direct monosynaptic projections from NRe to CA1 pyramidal cells, and only evidence of weak inputs to pyramidal cells in other areas of the hippocampal formation.

Results

NRe projections to hippocampal formation

We carried out stereotaxic injection of AAVs to express the light-sensitive channelrhodopsin2 variants Chronos or Chrimson [24] into NRe and prepared *ex vivo* brain slices in either coronal or horizontal planes to carry out patch clamp recordings targeting primarily prefrontal cortex and dorsal hippocampus, or entorhinal cortex and ventral hippocampus, respectively (example distribution of NRe axons in hippocampus shown in Fig. S1). As NRe sends a substantially denser projection to ventral over dorsal hippocampus [14], we first carried out patch clamp recordings in ventral CA1 (vCA1). We found that optogenetic activation of NRe axons in CA1 failed to elicit post-synaptic EPSCs in vCA1 pyramidal cells (Fig. 1B & 3A). We observed the same result in dorsal CA1 (dCA1) (Fig. 2B & 3A). In each slice tested, we only recorded a pyramidal cell as non-responsive when we could evoke a post-synaptic response in neurogliaform cells that, with their high NRe input probability acted as positive controls (Fig. 1D – F for vCA1 and Fig. 2C – E for dCA1; summary data Fig 3A). We found that there was no difference in the magnitude of NRe-EPSC on neurogliaform cells when evoked using either Chronos or Chrimson (Fig. S2). Additionally, we found no differences in NRe input probability or synaptic strength between dorsal and ventral hippocampus, nor did we observe differences in NRe-EPSC magnitude between MGE- and CGE-derived neurogliaform cells (Fig S3).

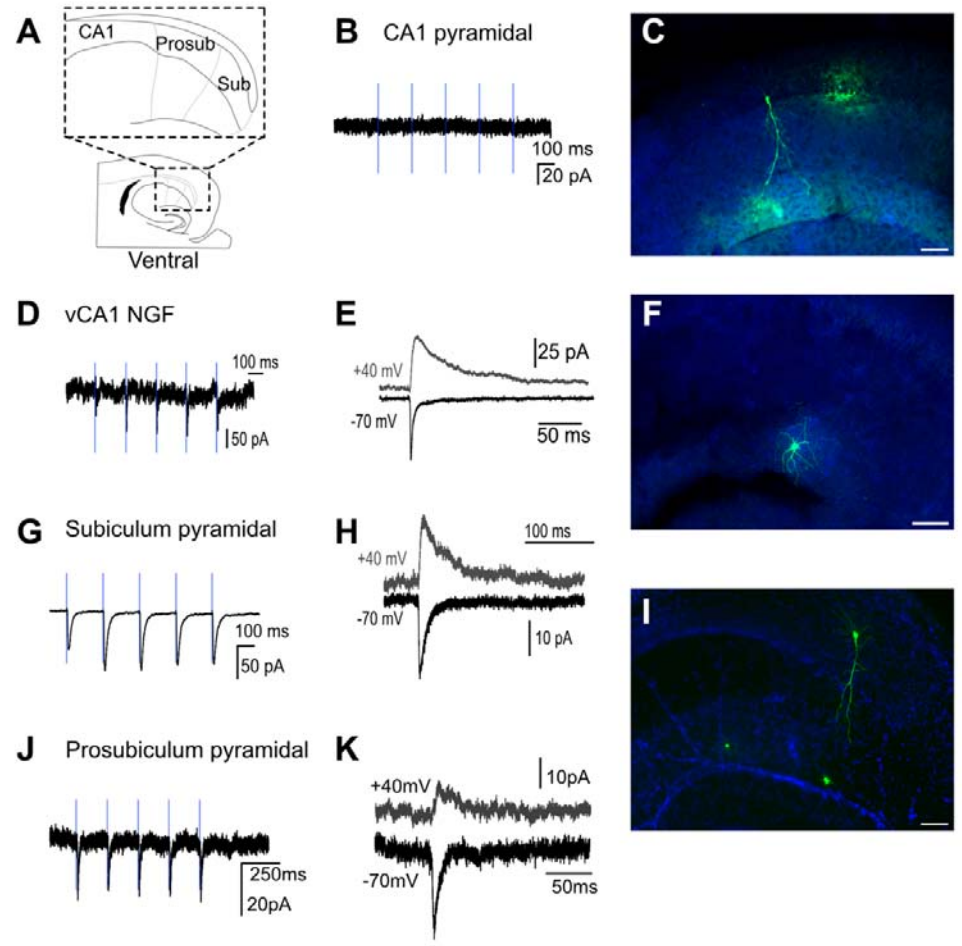

Figure 1: Prosubiculum pyramidal cells, unlike those in ventral CA1, do receive an input from NRe. Schematic representation of boundaries between dorsal CA1, prosubiculum and subiculum in horizontal orientation **A**, CA1 / prosubiculum boundary denoted as region where additional pyramidal cell layers arise 'below' tightly-packed layer that is a continuation of CA1 stratum pyramidale, after Lorento de No (1934). **B,** no response recorded from dCA1 pyramidal cell, **C,** *post hoc* recovery of a non-responsive pyramidal cell in dCA1, **D,** representative response trace from a ventral NGF. **E,** maximal AMPA and NMDA components of the responsive ventral NGF cell. **F,** A post hoc recovery from a ventral NGF cell. **G,** response trace from responsive pyramidal cell in dorsal subiculum. **H,** maximal AMPA and NMDA components of a responsive pyramidal cell from subiculum. **I,** post hoc recovery of a responsive pyramidal neuron in prosubiculum. **J,** a response trace from a responsive pyramidal cell in dorsal prosubiculum. **K,** maximal AMPA and NMDA components of a responsive pyramidal cell from prosubiculum. Scale bar 100 um.

Unlike CA1, we found that NRe projections to other parts of the hippocampus (prosubiculum and subiculum; Fig 1G – K for vCA1 and Fig 2F – H for dCA1) did indeed target principal cells although the synaptic strength of these inputs was low (-13.32±2.7 pA for subiculum;

summary data in Fig. 3), suggesting that NRe projections to hippocampal principal cells are either absent (CA1) or subthreshold (prosubiculum and subiculum Fig. 3), and unlikely to elicit firing. NRe projections to CA1 neurogliaform cells had a comparable magnitude of the AMPA-receptor mediated component of the EPSC (Fig. 3C) but the higher input resistance of inhibitory interneurons combined with the larger NMDA-AMPA ratio (Fig. 3E) would suggest that NRe could drive neurogliaform cells to spike, perhaps during periods of ongoing network activity.

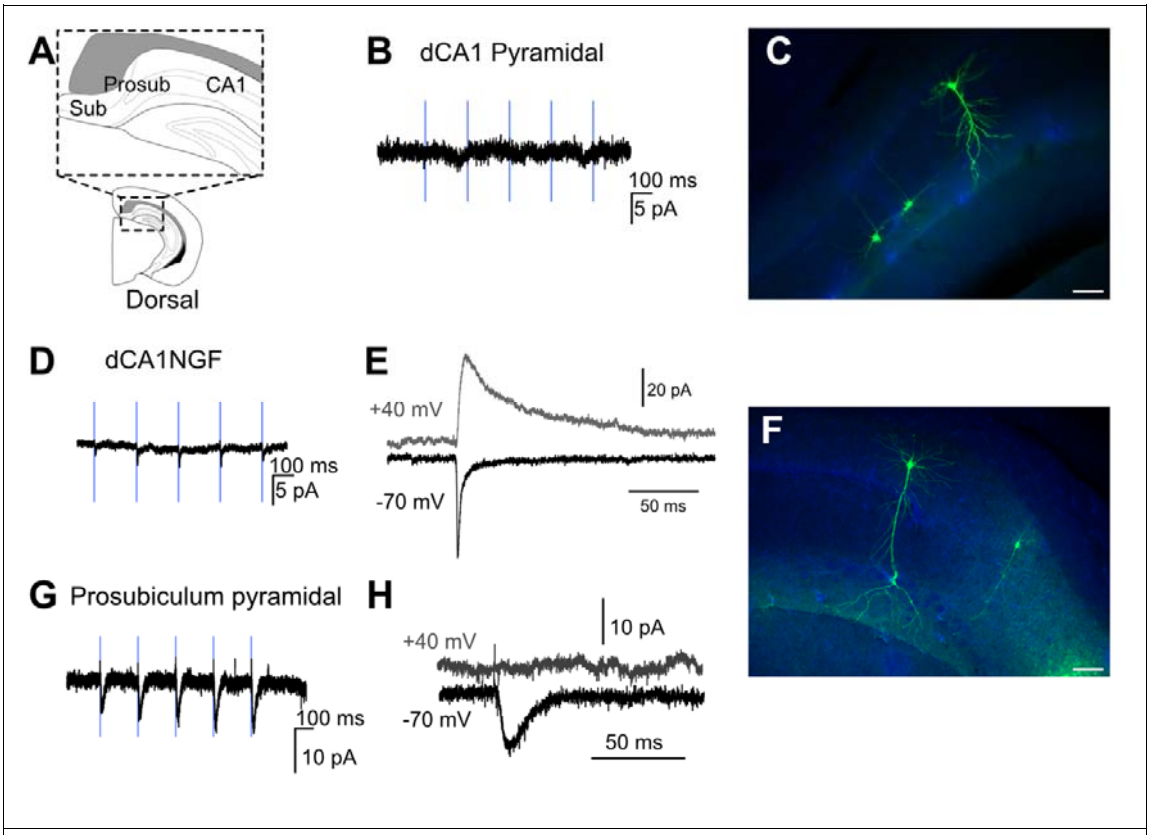

Figure 2: **Prosubiculum pyramidal cells, unlike those in dorsal CA1, do receive an input from NRe. A** Schematic representation of boundaries between dorsal CA1, prosubiculum and subiculum in coronal orientation, **B** a response trace from a non-responsive pyramidal neuron from dorsal CA1, **C** a *post hoc* recoveries of two neurogliaform cells and a non-responding CA1 pyramidal cell, **D** a representative response trace from a dorsal NGF, **E** maximal AMPA and NMDA components of a responsive NGF from dorsal CA1**, F** *post hoc* recoveries of two pyramidal cells, the non-responding CA1 pyramidal cell and responding pyramidal cell in the prosubiculum as well as a dorsal NGF, **G** a response trace from a responsive pyramidal neuron from dorsal prosubiculum, **H** maximal AMPA and NMDA components of a responsive pyramidal neuron from dorsal prosubiculum. Scale bar 100 um.

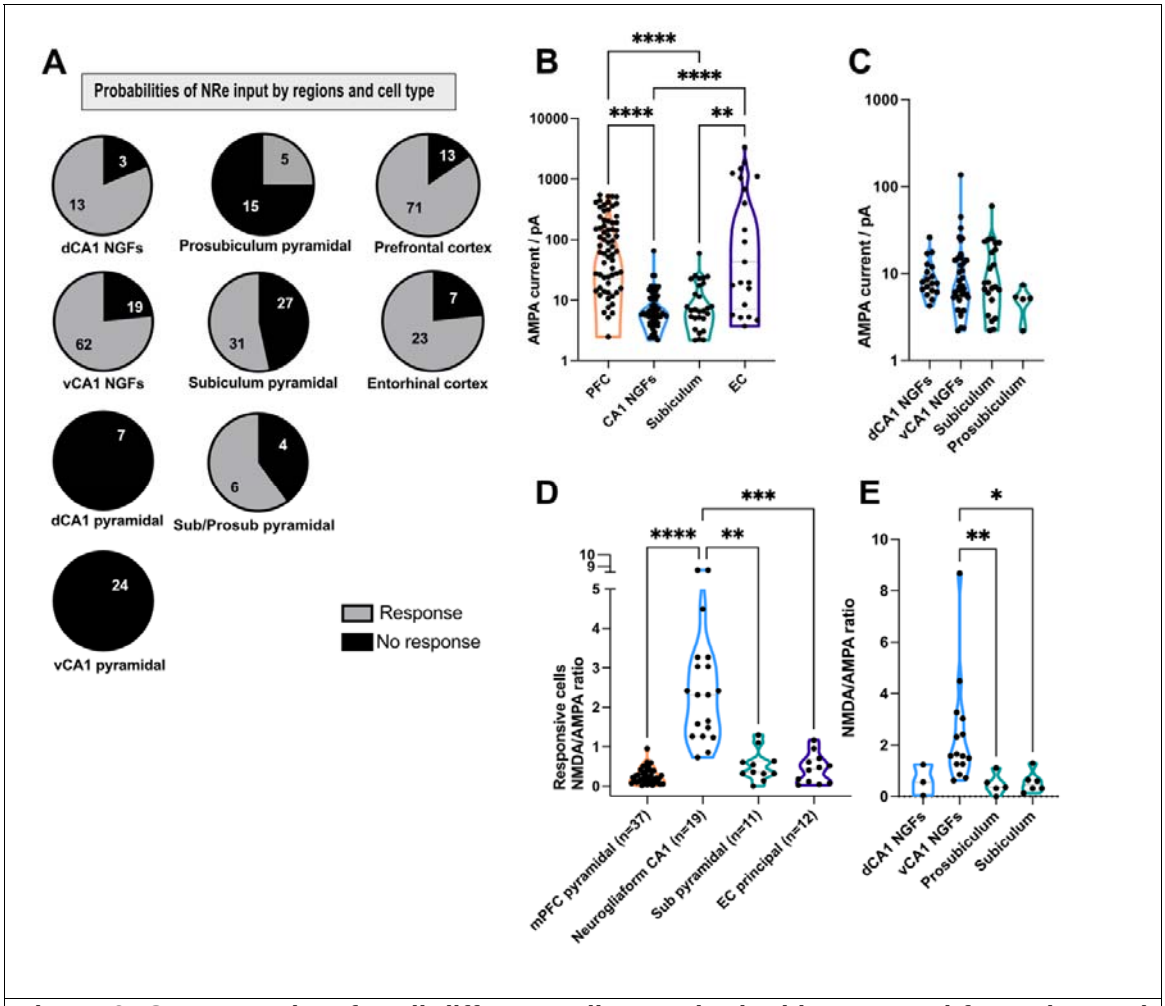

Figure 3: Summary data for all different cell types in the hippocampal formation and cortical areas. A NRe input probability for cells patched in hippocampus: dorsal NGFs (81.3%) and ventral NGFs (76.5%), dorsal (0%) and ventral (0%) CA1 pyramidal cells, prosubiculum (66.7%), subiculum (46.6%) and undetermined prosubiculum/subiculum (60%) pyramidal neurons, prefrontal cortex (84.5%) and entorhinal cortex (76.7%), **B** AMPA currents vary across different NRe target regions, with average AMPA currents being -142±19 pA for PFC pyramidal cells, -9.1±1.2 pA for NGFs, -12±2.3 pA for pyramidal cells in subiculum and -551±190 pA for principal cells in the entorhinal cortex. Kruskal-Wallis test (with Dunn's multiple comparison test) showed significant differences between CA1 NGFs vs EC ($p<0.0001$), CA1 NGFs vs PFC ($p<0.0001$), EC vs Subiculum ($p=0.0014$) and Subiculum vs PFC ($p<0.0001$), **C** AMPA currents across different regions with the average being -9.88±1.3 pA (dCA1 NGFs), -14.33±3.7pA (vCA1 NGFs), -13.32±2.7 pA (Subiculum pyramidal cells), -5.06±0.8 pA (Prosubiculum pyramidal cells), no significant difference found using a 2-way ANOVA with Tukey's multiple comparisons test, **D** NMDA/AMPA ratios of responding cells in different regions, average ratios were 1.1±0.26 (dCA1 NGFs), 3.8±1.7(vCA1 NGFs), 0.47±0.18 (Subiculum pyramidal cells), 0.55±0.17 (Prosubiculum pyramidal cells). Using a Kruskal-Wallis test with Dunn's multiple comparisons test significant different were found for NGFs vs Subiculum pyramidal cells ($p=0.0047$), EC principal vs NGFs ($p=0.0003$) and PFC pyramidal cells vs NGFs ($p<0.0001$), **E** Kruskal-Wallis test with Dunn's test for multiple comparisons, vNGFs cells have a significantly higher

NMDA/AMPA ration when compared to subiculum and prosubiculum pyramidal neurons (p=0.0206 and p=0.0074, respectively).

NRe projections to non-hippocampal areas

We also investigated the connectivity of the NRe to the cortical target regions, prefrontal (Fig. 4) and entorhinal cortices (Fig. 5). Similarly to the hippocampal region, the non-responding cells were only taken into the account if the same slice contained a positive control. Directly comparing NRe-evoked EPSC responses in cortical and hippocampal neurons, we found that principal cells in MEC and PFC pyramidal cells displayed significantly larger AMPA receptor-mediated EPSCs than subiculum pyramidal cells or CA1 NGFs (Fig. 3A, B & D). These data would suggest that, from a functional perspective, EC and PFC, and not the hippocampus, may be the principal targets of thalamic nucleus reuniens.

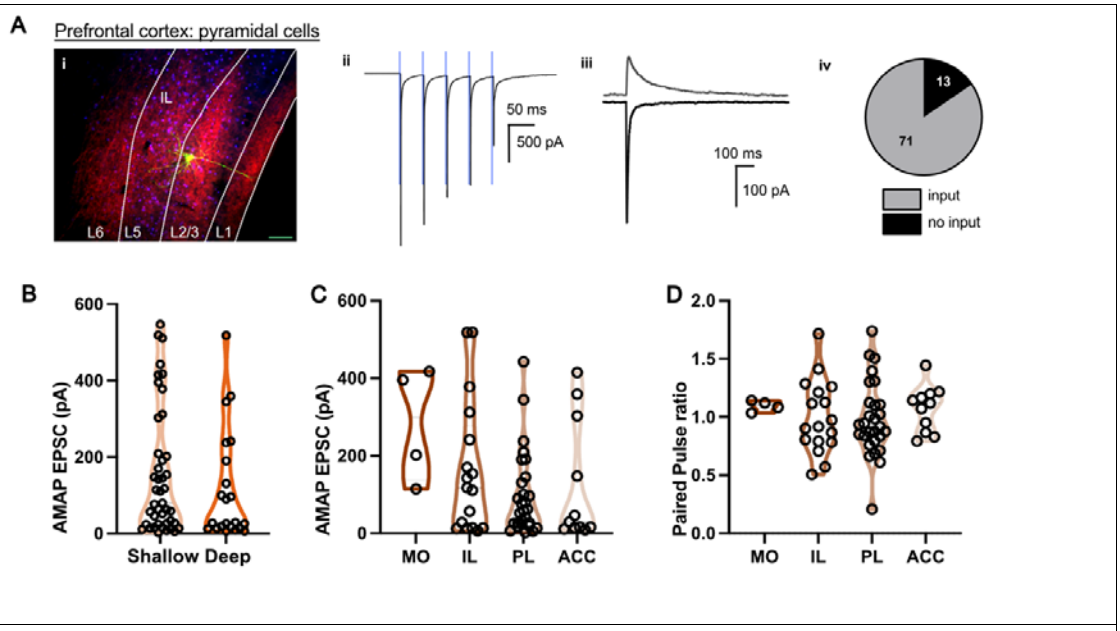

Figure 4: NRe-EPSCs are consistent across all subdivisions of the prefrontal cortex. A, A representative *post hoc* recovery of a pyramidal neuron in shallow layers of prefrontal cortex (**i**, scale bar 100-micron), optogenetic response trace (**ii**), AMPA and NMDA currents (**iii**) and input probability for patched pyramidal cells in prefrontal cortex (**iv**) **B**, AMPA-R mediated NRe-EPSC did not vary significant between deep (layer 2 to 3) and shallow (layer 5 to 6) pyramidal cells in PFC (shallow *vs* deep: 155 ± 25 pA (n=43) *vs* 114 ± 30 pA (n=22); p=0.232, Mann-Whitney test) **C**, AMPA-R mediated NRe-EPSC did not vary between different subdivisions of PFC (MO *vs* IL *vs* PL *vs* ACC: 282 ± 74 pA (n=4) *vs* 165 ± 42 pA (n=17) *vs* 98 ± 20 pA (n=28) *vs* 147 ± 56 pA (n=9 cells); p=0.46, one-way ANOVA) **D,** paired pulse ratio of AMPA-R mediated NRe-EPSC did not vary between different subdivisions of PFC (MO *vs* IL *vs* PL *vs* ACC: 1.1 ± 0.02 *vs* 1.0 ± 0.08

vs 0.98 ± 0.06 *vs* 1.1 ± 0.06; p=0.15, one-way ANOVA).

When looking at the prefrontal cortex overall, the majority of the pyramidal cells were responsive to the optogenetic stimulation (Fig. 3A& 4A). We saw a similar result in medial EC (Fig. 3A & 5A). We saw no difference in NRe-EPSC amplitude in PFC when comparing NRe input between medial orbital, infralimbic, prelimbic, and anterior cingulate areas (Fig. 4B-D). While there are at least two main principal cell subtypes in EC [25–27] , we did not parse EC neurons further into stellate or pyramidal cells so cannot comment on whether the large variability in NR-EPSC magnitude observed (Fig. 5D) corresponded to different subtypes of neuron. Nevertheless, these data confirm that NRe forms strong synaptic inputs onto principal neurons in both prefrontal and entorhinal areas.

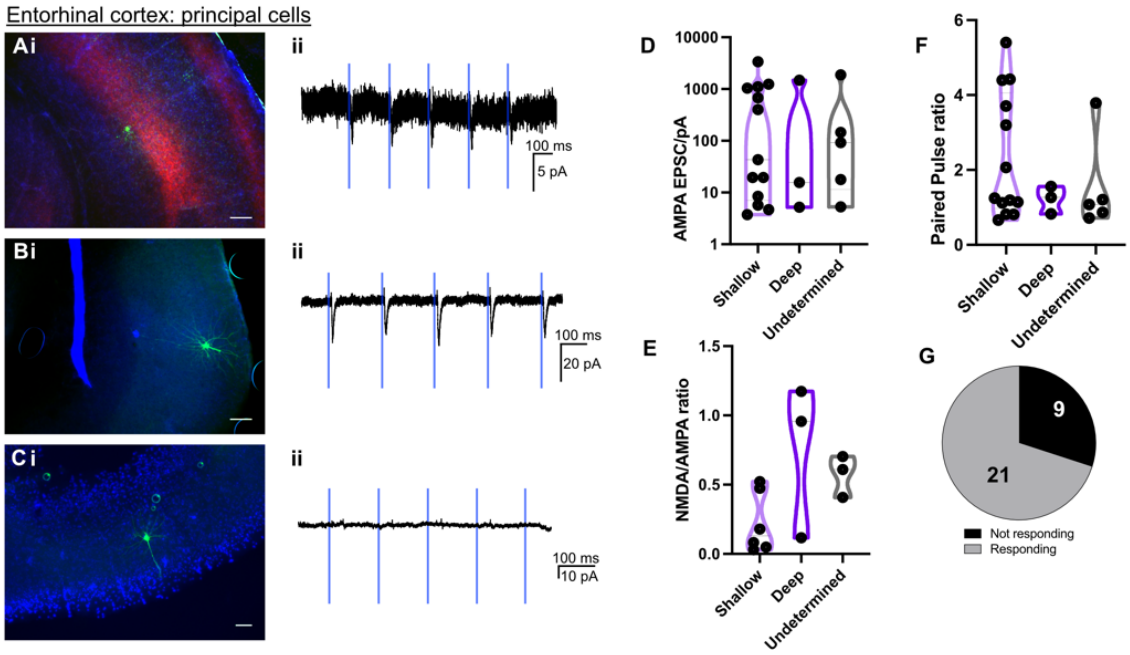

Figure 5: NRe-EPSCs are consistent across all subdivisions of the prefrontal cortex. A ,*Post hoc* recovery of layer 5 mEC pyramidal neuron (**i**) and optogenetic response trace (**ii**) **B**, *Post hoc* recovery of layer 2 mEC stellate cell (**i**) and optogenetic response trace (**ii**) **C**, *Post hoc* recovery of layer 2 mEC pyramidal neuron (**i**) and lack of response to optogenetic stimulus (**ii**) **D**, AMPA-R mediated NRe-EPSCs are not different in shallow vs deep layers of mEC **E**, NMDA/AMPA ratio in responsive neurons in shallow and deep layers of mEC is not significantly different **F**, Paired pulse ratio in responsive neurons in shallow and deep layers of mEC is not significantly different **G**, Input probability of mEC neurons.

Monosynaptic retrograde tracing from CA1 pyramidal cells

Although our patch-clamp electrophysiological recordings failed to find evidence of somatic EPSCs mediated by NRe inputs in CA1 pyramidal cells, given that NRe ESPCs in neurogliaform cells and subiculum pyramidal cells have a small magnitude (Fig. 3) we could not exclude the possibility that inputs were present but undetectable due to dendritic filtering of the NRe inputs arriving in the distal region of apical dendrites, despite having found no evidence of silent NMDA-R only synapses. Consequently, we carried out rabies-assisted monosynaptic circuit tracing in *Emx1-cre* mice that conditionally expressed the avian TVA receptor only in pyramidal cells (see methods & Fig. 6). We failed to find evidence of monosynaptic NRe inputs to pyramidal cells in either dorsal (Fig. 6A & D; n=6 mice) or ventral (Fig. 6 E&H, n=4) CA1 but, as expected, we saw consistent retrograde labelling in entorhinal cortices, medial and lateral septa and subiculum (we previously published some of this dataset in [8], confirming prior findings from Sun and colleagues [28]). We only observed retrograde labelling of NRe neurons when injections into ventral hippocampal formation included starter cells in both vCA1 and ventral prosubiculum / subiculum (Fig. 6I-J; n=3; all mice had retrogradely-labelled cells in NRe). To exclude the possibility that *Emx1* is not ubiquitously expressed throughout the hippocampus, we carried out a secondary analysis of RNAseq datasets publicly available from [29] and found that *Emx1* is indeed expressed throughout CA1 (Fig. S5).

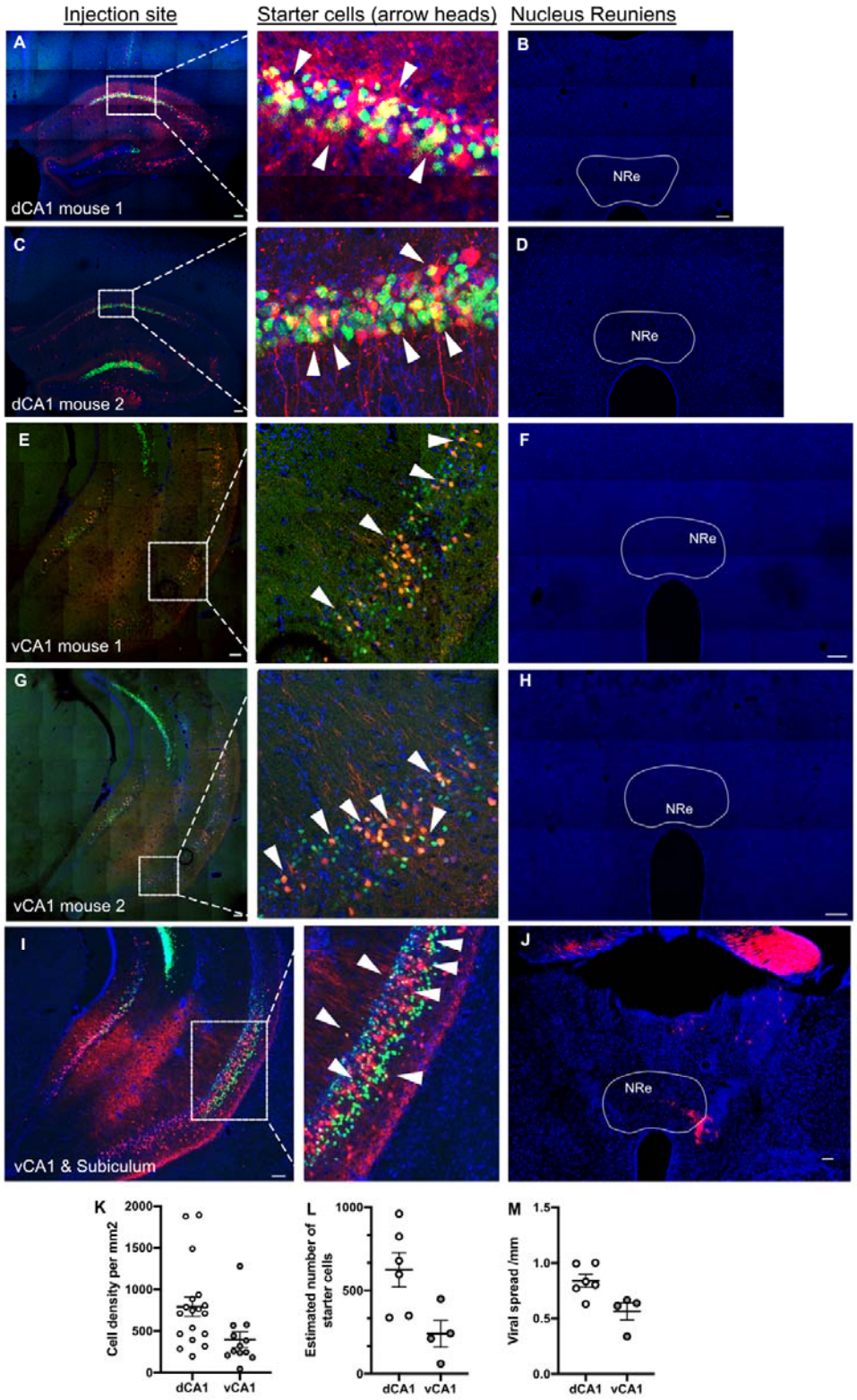

Figure 6: Monosynaptic retrograde tracing from hippocampus proper shows no cells originating from NRe, unless the starter cells are present in subiculum region. Injection site of a dCA1 injection: green cells contain helper virus and red cells are

pseudotyped rabies virus positive and starter cells (yellow) shown closer on the inset, shown by arrowheads **A&C**, No cells found in any thalamic section containing NRe **B&D**, Injection site of a vCA1 injection: green cells contain helper virus and red cells are pseudotyped rabies virus positive and starter cells (yellow) shown closer on the inset, shown by arrowheads **E&G,** No cells found in any thalamic section containing NRe **F&H,** Injection site of a vCA1 injection with virus spreading to subiculum: green cells contain helper virus and red cells are pseudotyped rabies virus positive and starter cells (yellow) shown closer on the inset, shown by arrowheads **I,** Cells and fibres seen in NRe as well as the fornix **J**, Cell density showing number of cells containing the pseudo-typed rabies virus in dorsal and ventral CA1 regions **K**, estimated number of starter cells, calculated from cell density and viral spread **L,** Viral spread distance in dorsal and ventral CA1 regions **M.** For more details on retrograde monosynaptic tracing experiment data see [8].

Discussion

Anatomical studies of NRe connectivity have led many to believe that the main function of NRe is to mediate prefrontal control over the hippocampus [e.g. 11]. However, our findings challenge this core assumption. We found that the magnitude of synaptic currents driven by NRe axons is at least an order of magnitude greater in EC and PFC than in either CA1 or subiculum. Furthermore, we have found that pyramidal cells in CA1 appear *not* receive monosynaptic inputs from NRe (determined using both anterograde optogenetic circuit-mapping and monosynaptic retrograde rabies tracing). It appears that NRe preferentially targets inhibitory interneurons in CA1 and pyramidal cells in prosubiculum.

Do CA1 pyramidal cells receive input from NRe?

Our data cannot entirely exclude the possibility of a monosynaptic projection from NRe to CA1 pyramidal cells, but if the connection does exist then it must be very sparse. Unlike hippocampus proper, we found that pyramidal cells in both prosubiculum and subiculum do indeed receive an input from NRe. The lack of input to pyramidal cells in our study was very specific to CA1; pyramidal cells in prosubiculum, identified using Lorente de Nó anatomical definitions [30], did receive direct input from NRe. While some regard prosubiculum as the distal region of CA1, recent transcriptomic studies confirm that it is distinct from both CA1 and subiculum [31], and our findings support this. It has been shown that NRe selectively innervates pyramidal cells in ventral subiculum that target nucleus accumbens and lateral habenula, whilst avoiding those that target PFC [32].

When we first reported these findings as a preprint in late 2021 [33], the results appeared controversial. Indeed, while a recent publication reported a direct monosynaptic projection from NRe to dorsal CA1 pyramidal cells [34], the key recordings in that study appear to have been in the subiculum and/or prosubiculum areas of the hippocampus rather than adjacent CA1 (see Figure 1 of this study - the only figure showing recording location). However, other published studies also provide support for our findings. An ultrastructural study reported that synapses from thalamic axons in CA1 make significantly fewer contacts per dendrite than those from entorhinal axons [35], supporting our electrophysiological data and the hypothesis that NRe input to hippocampus serves to modulate, rather than drive, HPC activity, perhaps via inhibitory tone in SL-M. A recent article from Leprince and colleagues [36] confirmed our main finding using a combination of *in vivo* and *ex vivo* approaches: they found that EC inputs strongly drive CA1 network activity in early postnatal circuitry and that, while NRe stimulation could drive network activity in CA1, this is almost entirely through inhibitory mechanisms. Indeed, this study reported that only 2 out of 26 CA1 pyramidal cells received input from NRe and it is unclear whether this connection persists into adulthood, with the authors speculating that it may be a transitory circuit in development that helps 'wire' the developing brain. This phenomenon has been widely observed in cortico-thalamic circuits, so this hypothesis is not without precedent (reviewed by [37]). Another recent *in vivo* study found that optogenetic inhibition of NRe projections to CA1 *increases* pyramidal cell activity [38], providing strong evidence to support our view of NRe's principal overall effect on hippocampal activity is one of inhibition and not excitation, even if some sparse monosynaptic projections to pyramidal cells persist into adulthood.

Circuit implications of the reuniens to hippocampus projection

In vivo electrophysiological studies looking at hippocampal LFPs found that only direct EC stimulation could drive a population spike in HPC, while NRe activation primarily evoked an LFP in SL-M [19,23], with the latter paper from this group arguing that low frequency stimulation of NRe likely promotes EC – HPC communication. These papers found that

stimulation of both EC and NRe lead to a supralinear summation of LFP amplitude in CA1, leading the authors to reasonably conclude that both regions drive excitation within CA1.

This work and other studies have now shown that the influence of NRe over CA1 is predominantly to inhibit hippocampal activity by targeting inhibitory interneurons. Neurogliaform cells, with a predominantly NMDA receptor-driven NRe-EPSC ([22] and the present study), will likely have a much stronger influence when hippocampus is already active. Given that EC projects to both DG and CA1, and that NRe evokes strong postsynaptic responses in EC neurons, we hypothesise that much of the effect of NRe activation on increasing the magnitude of LFP in CA1 is in fact an effect of feedforward excitation from entorhinal cortex. There is one report in the literature of midline thalamic stimulation inducing population spikes in CA1 [39], but, again, this could be due to feed-forward excitation from EC.

The NRe projection to CA1, targeting neurogliaform cells, could serve to inhibit distal dendrites of selected CA1 pyramidal cell ensembles and increase the fidelity of EC – HPC communication. Another recent study, using *in vivo* juxtacellular recording in mice, found that neurogliaform cells in CA1 can decouple pyramidal cells from entorhinal-driven gamma oscillations without altering the firing rates of pyramidal cells [40]. Given that NRe projections to CA1 are highly selective for neurogliaform cells ([22,36] and the present study), we have previously suggested that the NRe recruitment of neurogliaform cells allows NRe to reroute information flow through the hippocampus, switching between memory encoding and memory retrieval [41].

Implications for NRe function?

Prefrontal cortex projections to the hippocampus are important for impulse control [42], and lesions of NRe inhibit this function [43]. A recent suggestion for the function of the prefrontal – reuniens – hippocampal circuit is that it actively suppress ongoing memory retrieval [44],

and our study provides a mechanism through which this could occur. Indeed, the large NMDA:AMPA ratio of NRe-EPSCs in neurogliaform cells that we reported here and previously [22] suggest that the inhibitory influence evoked via NRe activation is much more likely to be effective in the context of ongoing network activity. Recent behavioural data suggest the requirement for NRe in spatial memory retrieval or “online” spatial processing, but not for off-line consolidation or long-term storage [45]. Given that the largest NRe-mediated EPSCs were present in entorhinal and prefrontal cortices, it could be that NRe suppresses hippocampal activation to facilitate prefrontal – entorhinal communication during associative memory consolidation [6]. Alternatively, an inhibitory influence of NRe over hippocampus has been proposed as the mechanism through which prefrontal cortex can prevent retrieval, e.g. during extinction of fear memory [46].

Given the large NMDA receptor-mediated component of NRe-EPSCs onto neurogliaform cells, one is tempted to suggest that the role of NRe could be to increase Ca^{2+} in NGF dendrites to activate a NO-dependent suppression of inhibition [47] that could serve to enhance entorhinal-to-CA1 communication. Future behavioural experiments are required to test the function of NRe to CA1 projections, perhaps by using retrograde viruses to allow specific targeting only of those NRe neurons that project directly to CA1.

Concluding remarks

We have found that, unique to all hippocampal or cortical regions, pyramidal cells in hippocampal region CA1 do not receive monosynaptic input from the thalamus. Even in parts of the hippocampal formation where principal cells do receive direct input from reuniens, the amplitude of the evoked currents is small and likely to be modulatory in nature. These surprise findings suggest that the NRe to hippocampus projection may be a bit of a ‘red herring’ for developing conceptual models of NRe function. Given that functional interactions between PFC and HPC are well-defined, our study raises one very important

question. In the absence of a direct PFC to HPC projection, how can PFC exert influence of hippocampal circuitry? Does NRe have no role in this at all, or are indirect pathways (e.g. via entorhinal cortex) involved? We suspect that the answer may be the latter, and future research should focus on the NRe innervation of prefrontal and entorhinal regions.

Materials and Methods

Animals

All experiments were conducted in accordance with animal protocols approved by the National Institutes of Health, or in accordance with the UK Animals (Scientific Procedures) Act 1986 after local ethical review by the Animal Welfare and Ethical Review Board at the University of Exeter. We used *Nkx2-1-cre* [48]:RCE or *Nkx2-1-cre:*Ai9 and *Htr3a*-GFP [49] mice to target interneurons of MGE or CGE origin, respectively, in electrophysiological experiments. *Nkx2-1-cre* mice were obtained from Jackson laboratories (C57BL/6J-Tg(Nkx2-1-cre)2Sand/J, stock number 008661) and Htr3a-GFP mice (Tg(Htr3a-EGFP)DH30Gsat) were cryo-recovered from MMRRC (NC, USA) and back-crossed onto C57BL/6J mice (Charles River, UK). We used *Emx1*-cre mice [50] crossed with floxed TVA mice [51] to allow specific targeting of pyramidal cells for monosynaptic rabies tracing. *Emx1*-cre mice were obtained from Jackson laboratories (B6.129S2-Emx1$^{tm1(cre)Krj}$, stock number 005628) and floxed TVA mice (LSL-R26$^{Tva\text{-}lacZ}$) were kindly provided by Prof Dieter Saur (Technical University of Munich, Germany). All animals were maintained on a 12 h constant light / dark cycle and had access to food and water *ad libitum* and were grouped housed wherever possible. We used standard enrichment that included cardboard tubes, wooden chew blocks and nesting material.

Drugs and chemicals

CGP55845, DNQX, DL-AP5 and picrotoxin were purchased from Tocris Bioscience, and all other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Stereotaxic injections for electrophysiology experiments

For optogenetic experiments, we used *Nkx2-1*-cre:Ai9, *Nkx2-1-cre*:RCE or *Htr3a*-GFP mice of both sexes, totalling at 65 mice. with the age at the time of stereotaxic injection ranging from 2 – 7 months. Mice of both sexes were used for stereotaxic surgery. Mean weight of the mouse prior to stereotaxic surgery was 26 g (ranging from 17.5 g to 49.5 g). Two different viruses were used for stereotaxic surgeries: AAV8-hSyn-Chrimson-TdTom (UNC Viral Vector Core, USA, contributed by Ed Boyden; titre 6.3×10^{12} viral particles / ml). AAV8-hSyn-Chronos-GFP (UNC Viral Vector Core, USA. contributed by Ed Boyden; titre 3.1×10^{13} viral particles / ml).

For the surgery, the mice were anaesthetised with 5% isoflurane and anaesthesia was maintained with use of 1.5 to 2.5% isoflurane (flow rate of ~2 $Lmin^{-1}$ O_2). The mice were placed on a heated pad (37 C) for the duration of the surgery and given 0.1 mg/kg of buprenorphine (buprenorphine hydrochloride, Henry Schein) subcutaneously at the start of surgery as an adjunct analgesic, plus carprofen 1 mg/kg (Rimadyl, Henry Schein) was given at a dose of 5 mg/kg subcutaneously post-surgery and on subsequent days, as required. To target nucleus reuniens, we used the following coordinates: A/P -0.8 mm, M/L 0.0 mm, D/V 3.8 mm from pia, with 300 nl of virus (infused at 100 nl min^{-1}). After the surgery, the mice were allowed at least a 3-week recovery period to allow sufficient time for the expression of the viral construct. For whole cell patch clamping experiments AAV8-hSyn-Chronos-GFP or AAV8-hSyn-Chrimson-TdTom were used for *Nkx2.1*-cre:Ai9 or *Htr3a*-GFP mice, respectively, although a small number of *Htr3a*-GFP mice received AAV8-hSyn-Chronos-GFP to allow direct comparison of EPSC amplitude in the same population of neurons.

Monosynaptic retrograde tracing

For anatomical experiments we used the monosynaptic rabies tracing method that has been previously reported by others [28]. Two mouse lines (*Emx1*-cre and floxed TVA) were

crossed together in order to ensure that the modified rabies virus only targets the pyramidal cells, with *Emx1-cre* mice used as controls to ensure the rabies virus did not transduce neurons in the absence of TVA. A total of 22 mice of both sexes were used, with 2 mice being excluded from the analysis due to failed injections. The age of the mice used ranged from 2 to 6 months, with pre-surgical weights from 18.9 g to 40.6 g (mean age 3.5 months, mean weight 24.6 g). To highlight the efferent projections from nucleus reuniens to hippocampus, we elected to inject into dorsal and ventral CA1 using the following coordinates: dCA1 was targeted at A/P -2 mm (relative to Bregma), M/L -1.5 mm and D/V-1.35 mm (from pia) and vCA1 at A/P -2.8 mm (relative to Bregma), M/L -2.4 mm and D/V-4.2 mm (from pia). The stereotaxic injections of AAV8-FLEX-H2B-GFP-2A-oG (Provided by John Naughton at Salk Institute Viral Vector Core, USA, titre 3.93×10^{12} viral particles / ml), followed by injection EnvA G-deleted Rabies-mCherry (Provided by John Naughton at Salk Institute Viral Vector Core, titre 6.13×10^{8} viral particles / ml; or from Viral Vector Core facility of the Kavli Institute for Systems Neuroscience, NTNU, Norway, titre 2.6×10^{10} viral particles / ml) 2 weeks after the initial viral injection were performed in the right hemisphere only. The mice were maintained for 2 weeks to provide optimal time for expression, and were killed by transcardial perfusion / fixation with 4% paraformaldehyde (Cat number P6148 Sigma-Aldrich, UK) in 0.1 M phosphate buffer.

Following the transcardial perfusion, the brains were dissected out and post-fixed for 24 h in 4% pfa solution, after which they were cryoprotected using the 30% sucrose in PBS solution. Once cryoprotected, the brains were sliced at 50 microns using the freezing microtome (Leica, SM2010 R). Selected slices (1 in 5 serially, increasing to 1 in 3 between -0.5 and -1.8 Bregma to ensure thorough representation of nucleus Reuniens of the thalamus across the anterior-posterior axes) were mounted using the Hard Set mounting medium with DAPI (Vectashield, Vector Lab, H-1500-10) and the fluorescent fibres were visualised with CoolLED on Nikkon 800 microscope. Representative photos of projections patterns can be found on figure 1 – figure supplement 1.

Slice preparation and electrophysiology

A minimum of 3 weeks recovery period following the stereotaxic surgery was allowed. Mice were anesthetised with isoflurane and the brain was rapidly dissected out in room temperature NMDG cutting solution, containing (in mM): 135 NMDG, 20 Choline bicarbonate, 10 glucose, 1.5 $MgCl_2$, 1.2 KH_2PO_4, 1 KCl, 0.5 $CaCl_2$, saturated with 95% O_2 and 5% CO_2 (pH 7.3-7.4). Coronal or horizontal slices (400 um) were cut to target prefrontal cortex and dorsal CA1 vs ventral CA1, subiculum and entorhinal cortex, respectively, using a VT-1200S vibratome (Leica Microsystems, Germany). Afterwards, the slices were transferred into a chamber containing recording aCSF, composed of (in mM): 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1.25 NaH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgCl_2$, and 10 glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4) and placed in a water bath at 37 °C for 30 minutes, following which they were kept at room temperature until recording.

For recordings individual slices were attached onto 0.1% poly-L-lysine (Sigma Aldrich, P8920) coated glass slides and placed into the upright microscope and visualized using infrared differential interference contrast microscopy (Olympus BX51 or Scientifica SliceScope). CoolLED pE-4000 system was used to visualise the fibres as well as interneurons, and to provide optogenetic stimulation. The slices were submerged in recording aCSF, warmed to 32-34 °C, and the rate of perfusion was kept at 5ml/min. The recording electrodes were typically 3-5 MΩ size and were pulled from borosilicate glass (World Precision Instruments). The intracellular solution used had the following composition (in mM): 135 Cs-methanesulfonate, 8 NaCl, 10 HEPES, 0.5 EGTA, 4 MgATP, 0.3 Na-GTP, 5 QX314, plus 2 mg/ml biocytin (VWR International, UK), at pH 7.25 adjusted with CsOH and 285 mOsm.

A train stimulation with 5 pulses of 470 nm or 660 nm was used to excite the Chronos [52] or Chrimson opsins, respectively. The presence or absence of responses was recorded in

voltage clamp mode. Cells that were found to have a response to a train stimulation were then switched onto repeated single pulse protocol (ISI of 10 s), and the AMPA response was recorded at a holding potential of -70 mV. GABA-R antagonists were bath applied from the start in hippocampus and subiculum, but not in EC or PFC due to epileptiform activity being observed upon NRe stimulation with GABA-R antagonists present. The extracellular $GABA_A$ and $GABA_B$ receptor antagonists used were picrotoxin (100 µM) and CGP55845 (1 µM). 10 µm of DNQX was added to abolish the AMPA current at -70 mV, after which the cell was switched to +40 mV to record the NMDA current. To confirm the identity of NMDA current, D-AP 5 (100 µM) was added at the end of the recording. Whole-cell patch-clamp recordings were made using a Multiclamp 700A or 700B amplifier (Molecular Devices, Sunnyvale, CA). Signals were filtered at 3 kHz and digitized at 10 kHz using a Digidata 1322A or 1440A and pClamp 9.2 or 10.2 (Molecular Devices, USA). Recordings were not corrected for a liquid junction potential. The recordings were then imported into IgorPro (Wavemetrics, OR) using Neuromatic (Thinkrandom, UK) for further analysis.

Post hoc morphological recovery

The tissue slices were post-fixed in 4% PFA solution for an hour after patch clamping recordings and then transferred to PBS (Melford, UK). Slices were washed thrice in 0.1 M PBS solution, followed by three washes in PBS with 0.5% Triton X (Sigma, UK). Two blocking steps were used: 100mM of Glycine (Sigma, UK) incubation for 20 mins and then 1-hour long incubation with blocking buffer with 5% goat serum (VectorLabs, UK) at room temperature. Incubation with streptavidin (1:500 dilution, VectorLabs, UK) was done for 2 hours at RT in a carrier solution consisting of 5% goat serum and PBS. Finally, the slices were washed in PBS and transferred to 30% sucrose (Thermofisher, UK) until cryoprotected. Then slices were re-sectioned at 100-micron thickness using SM2010R freezing microtome (Leica, UK)) at -20°C and mounted onto glass-slides and HardSet mounting medium with DAPI was used (VectorLabs, UK).

Data Analysis

For quality control, cells with changes in input resistance of over 20% throughout the course of the experiment were excluded from the data analysis. The AMPA receptor-mediated EPSC was determined as the maximal EPSC peak at -70mV and NMDA receptor-mediated EPSC as the highest peak at +40 mV. GraphPad Prism (GraphPad, CA) was used for statistical analysis. Data were tested for normality using the D'Agostino and Pearson test and subsequently analysed by parametric or nonparametric tests as appropriate. Unless otherwise stated, all values are mean ± SEM. RNAseq data from [29] were downloaded from NCBI Gene Expression omnibus [53], accession GSE67403) and analysed in R Statistical Software v4.3.1 [54] and RStudio v 2023.6.1.524 [55], using the tidyverse [56] and ggplot2 packages [57].

Acknowledgements

This work was supported by Biotechnology and Biological Sciences Research Council grant BB/P001475/1 (MTC) and an NIH intramural award (CJM). Salary support from SK was provided by Alzheimer's Research UK Interdisciplinary research grant ARUK-IRG2017B-4 (MTC). GMS and ESB are both GW4 BioMed doctoral training program students funded by the Medical Research Council (MR/N0137941/1). We are grateful to Prof John Aggleton (University of Cardiff) for advice and discussions on anatomy. We gratefully acknowledge the support of the Inger M Lilya and George Simpson Biological Psychiatry Scholarships. For the purpose of open access, the authors have applied a Creative Commons Attribution (CC-BY) licence to any Author Accepted Manuscript version arising from this submission.

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