Hippocampal CA1 pyramidal cells do not receive monosynaptic input from thalamic nucleus reuniens

Lilya Andrianova[1,2], Erica S Brady[1], Gabriella Margetts-Smith[1], Shivali Kohli[1], Chris J McBain[3] and Michael T Craig[1,2,3]

1. Institute of Biomedical and Clinical Science, University of Exeter Medical School, Prince of Wales Road, Exeter, EX4 4PS, UK
2. Institute of Neuroscience and Psychology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK.
3. Program in Developmental Neurobiology, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA

Correspondence to Michael T Craig, mick.craig@glasgow.ac.uk

Thalamic nucleus reuniens (NRe) is located on the midline and acts as a hub to mediate interactions between hippocampal, entorhinal and prefrontal areas although these connections are poorly defined at the cellular and synaptic level. Using electrophysiology and monosynaptic circuit-tracing, we found that pyramidal cells in CA1 receive no direct input from NRe. This contrasts starkly with prefrontal cortex, subiculum and entorhinal cortex, and indicates that NRe inputs to CA1 primarily drive local inhibition and not excitation they do in the other regions.

Nucleus reuniens (NRe) is a midline thalamic nucleus that forms reciprocal connections with medial prefrontal cortex (mPFC) and subiculum, while also projecting to hippocampal region CA1 and entorhinal cortex (EC)[1,2], thereby providing the principal subcortical relay through which mPFC communicates with the hippocampus proper. Although electrical stimulation in NRe fails to elicit spiking in the CA1 pyramidal layer *in vivo*[3], although it is assumed that NRe targets CA1 pyramidal cells[1]. This assumption is reasonable as we are unaware of a cortical region in which thalamic inputs do *not* target pyramidal cells. Surprisingly, despite NRe's important role in goal-directed spatial navigation[4] and working memory[5], NRe projections to CA1 remain 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[6]. Optogenetic stimulation of NRe inputs to these neurogliaform cells elicits monosynaptic EPSCs that are defined by large NMDA receptor-mediated components.[6] We hypothesised that a similarly large NMDA-R component in pyramidal cells could underlie greatly enhanced NRe-fEPSPs in CA1 during EC input coactivation[7].

Remarkably, optogenetic activation of NRe axons in CA1 (representative images of axons in supplementary figure 1) using the light-sensitive tools Chronos or Chrimson[8] (supplementary figure 2) failed to elicit post-synaptic EPSCs in CA1 pyramidal cells (figure 1A & F), in either ventral CA1 (vCA1; n= 24, supplementary figure 3) or dorsal CA1 (dCA1; n=7,

supplementary figure 3). In each slice tested, we only counted an input as negative when we could evoke a

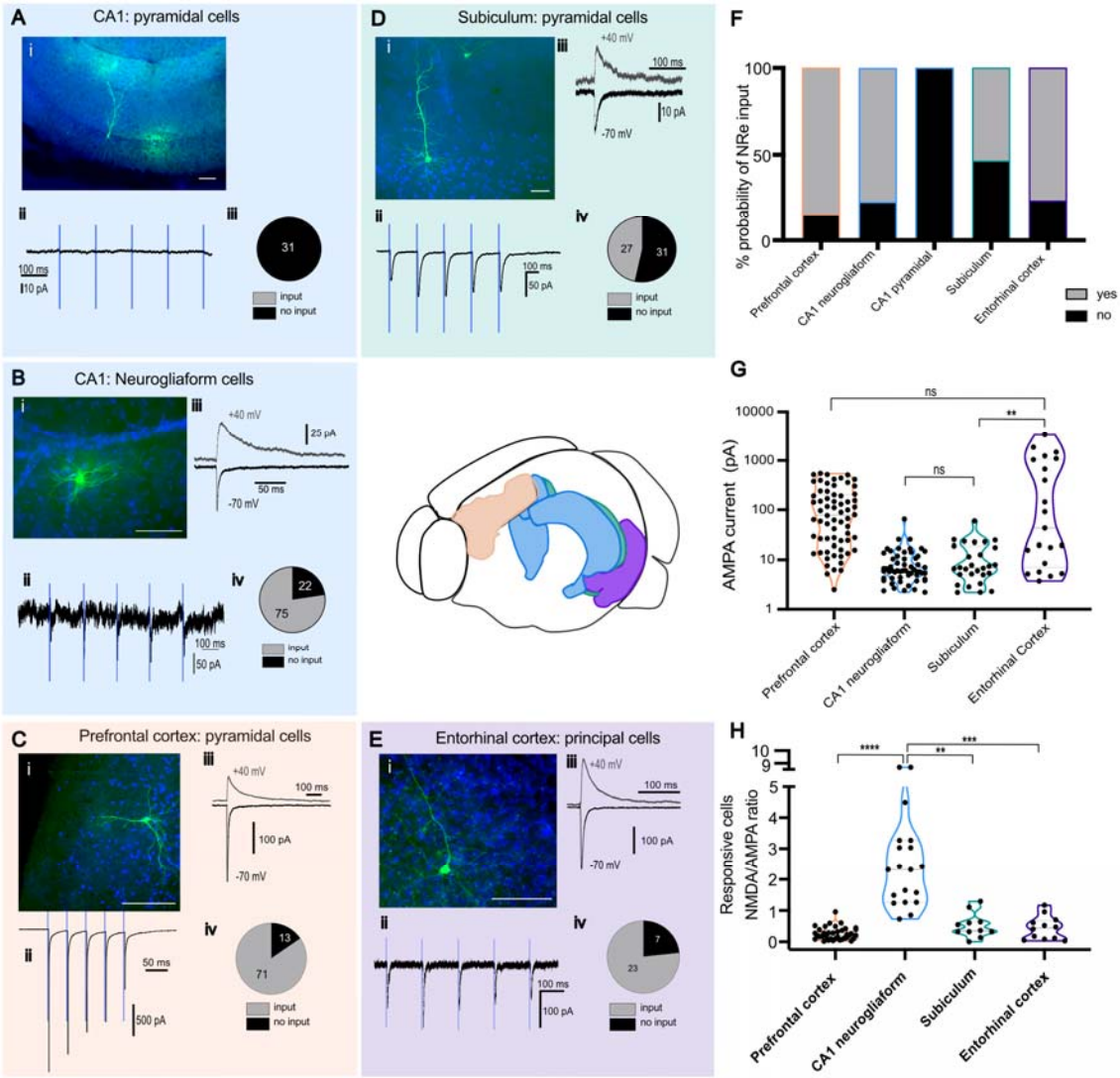

Figure 1: NRe inputs into downstream regions. Example *post hoc* cell recoveries (i), NRe-EPSCs at 5 Hz (ii), NRe AMPA-R and NMDA-R-mediated EPSCs (iii) and connection probability (iv) for **A,** CA1 pyramidal cells; **B**, CA1 neurogliaform cells (NGFs); **C**, prefrontal cortex pyramidal cells; **D**, subiculum pyramidal cells; **E**, Entorhinal cortex principal cells (PCs). **F**, NRe input probability across regions. **G**, mean NRe-AMPA-R ESPCs: PFC pyr, 142±19 pA (n=66); CA1 NGF, 9.1±1.2 pA (n=57); Subiculum pyr, 12±2.3 pA (n=28); EC PCs, 551±190 pA for EC (n=21), all pair-wise comparisons are significantly different at p<0.0001, unless otherwise shown; p<0.0001 using Kruskal-Wallis (Dunn's multiple comparisons test). **H,** mean NRe-EPSC NMDA/AMPA ratios: PFC pyr, 0.26±0.03 (n=37); CA1 NGF, 2.8±0.5 (n=19); subiculum pyr; 0.52±0.1 (n=11); EC PC, 0.44±0.1 (n=12), ns between PFC, Subiculum and EC; ** - p=0.0047, *** - 0.0003, ****- p<0.0001 using Kruskal-Wallis (Dunn's multiple comparisons test). Colours used in schematic in centre of figure correspond with those used in other panels.

post-synaptic response in neurogliaform cells that, with their high NRe input probability, acted as positive controls (figure 1B & F – H & supplementary figure 4). In contrast, optogenetic

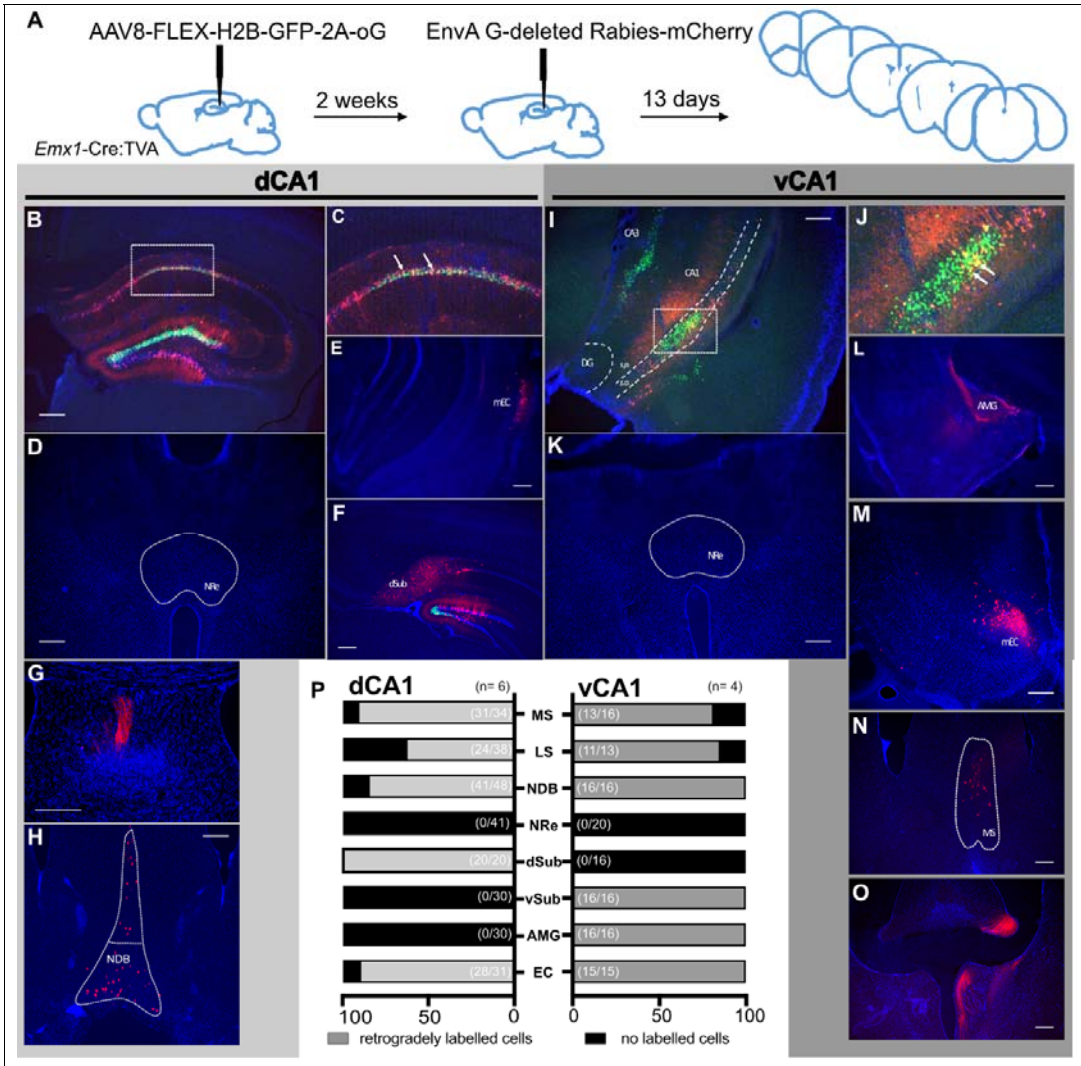

Figure 2: Monosynaptic retrograde tracing from hippocampus proper. A, schematic of experimental protocol. **B & C**, dorsal CA1 injection site, showing starter cells in yellow. Representative images showing: **D**, absence of retrogradely-labelled somata or processes in NRe; presence of retrogradely-labelled cells (red) in **E,** mEC; **F** dorsal subiculum; **G,** dorsal fornix (fibres only); **H,** medial and lateral septa. **I & J,** ventral CA1 injection site with starter cells (yellow). Representative images showing: **K**, absence of retrogradely-labelled somata or processes in NRe; presence of retrogradely-labelled cells (red) in **L,** amygdala; **M**, entorhinal cortex; **N**, medial septum; **O**, fimbria (fibres only. **P**, summary of data; bar graphs show the percentage of sections from each animal showing labelled presynaptic neurons while numbers in brackets show number of sections with labelled cells vs the total number of cells examined. dCA1, n=6 mice; vCA1, n=4 mice.

stimulation of midline thalamic projections to all other postsynaptic targets of NRe input consistently evoked EPSCs in glutamatergic neurons, with mEC principal cells and PFC

pyramidal cells displaying significantly larger AMPA receptor-mediated EPSCs than subiculum pyramidal cells or CA1 NGFs (figure 1 C – H). Pyramidal cells in prosubiculum also displayed small NRe-EPSCs in response to optogenetic stimulation, confirming a direct NRe input (supplementary figure 4). We saw no difference in NRe-EPSC amplitude in PFC when comparing NRe input between medial orbtial, infralimbic, prelimbic and anterior cingulate areas (supplementary figure 5).

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 (figure 1G) 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, although we 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 & figure 2). We failed to find evidence of monosynaptic NRe inputs to pyramidal cells in either dorsal (figure 2 B, C & P; n=6 mice) or ventral (figure 2 I, J & P, n=4) CA1 but, as expected, we saw consistent retrograde labelling in entorhinal cortices (figure 2 E, M & P), medial and lateral septa (figure 2 H, N & P) and subiculum (figure 2 F & P; after Sun and colleagues[9]). We only observed retrograde labelling of NRe neurons when injections into ventral hippocampal formation included starter cells in both vCA1 and ventral prosubiculum / subiculum (supplementary figure 6 E to I; n=3; all mice had retrogradely-labelled cells in NRe).

Our findings indicate that NRe does not form monosynaptic projections to pyramidal cells in the CA1, contrasting greatly with the rest of the prefrontal-hippocampal-entorhinal circuit. This unexpected result contradicts widely-held assumptions about interactions between NRe and hippocampus[1,10] and is inconsistent with a recent study that used ultrastructural data to show that midline thalamic inputs to CA1 formed ‘simple’ (and presumably weak) synapses

on distal dendrites of dCA1 pyramidal cells[11]. We cannot conclusively state the reason for this discrepancy, although the lack of input to pyramidal cells in our study was very specific to CA1; pyramidal cells in prosubiculum, using Lorente de Nó anatomical definitions[12], did receive direct input from NRe (supplementary figure 3). While some regard prosubiculum as the distal region of CA1, recent transcriptomic studies confirm that it is distinct from both CA1 and subiculum[13], and our findings support this. There is one report in the literature of midline thalamic stimulation inducing population spikes in CA1[14], but this could be due to feed-forward excitation from mEC. Our data strongly support the assertion that CA1 pyramidal cells are not the principal target of NRe projections, and that the mechanism through which mPFC exerts control over CA1 is by activating inhibitory interneurons. So, what is the function of this projection? Prefrontal cortex projections to the hippocampus are important for impulse control[15], and lesions of NRe inhibit this function[16]. A recent suggestion for the function of the prefrontal – reuniens – hippocampal circuit is that it actively suppress ongoing memory retrieval[17], 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[6], 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[18]. 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[19]. 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.

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