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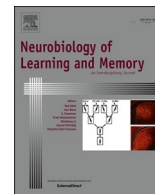
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## Neurobiology of Learning and Memory

journal homepage: [www.elsevier.com/locate/ynlme](http://www.elsevier.com/locate/ynlme)GluA1-containing AMPA receptors are necessary for sparse memory engram formation<sup>☆</sup>Thije S. Willems<sup>a,1</sup>, Hui Xiong<sup>a,b,1</sup>, Helmut W. Kessels<sup>a</sup>, Sylvie L. Lesuis<sup>a,\*</sup><sup>a</sup> Department of Cell and Circuit Neuroscience, Swammerdam Institute for Life Sciences, Amsterdam Neuroscience, University of Amsterdam, Amsterdam, the Netherlands<sup>b</sup> Westlake Laboratory of Life Sciences and Biomedicine, Hangzhou, Zhejiang 310024, China

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## ABSTRACT

Memory formation depends on the selective recruitment of neuronal ensembles into circuits known as engrams, which represent the physical substrate of memory. Sparse encoding of these ensembles is essential for memory specificity and efficiency. AMPA receptor (AMPA) subunits, particularly GluA1, play a central role in synaptic plasticity, which underpins memory encoding. This study investigates how GluA1 expression influences the recruitment of neurons into memory engrams. Using global GluA1 knockout (GluA1<sup>KO</sup>) mice, localized knockout models, and contextual fear-conditioning paradigms, we evaluated the role of GluA1 in memory formation and engram sparsity.

GluA1<sup>KO</sup> mice exhibited impaired short-term memory retention but preserved 24-hour contextual memory. Despite this, these mice displayed increased expression of the immediate early gene Arc in hippocampal neurons, indicative of a denser engram network. Electrophysiological analyses revealed reduced synaptic strength in GluA1-deficient neurons, irrespective of Arc expression. Localized GluA1 knockout in the hippocampus confirmed that GluA1 deficiency increases neuronal recruitment into engrams, disrupting the sparse encoding typically observed in wild-type mice.

These findings demonstrate that GluA1-containing AMPARs constrain engram size, ensuring selective recruitment of neurons for efficient memory encoding. By regulating synaptic plasticity, GluA1 facilitates both the encoding and size of memory circuits. This study highlights the critical role of GluA1 in maintaining sparse engram formation and provides insight into mechanisms underlying memory deficits in conditions where synaptic composition is altered.

## 1. Introduction

Memory formation relies on the recruitment of specific neuronal ensembles into stable circuits known as memory engrams. These ensembles are thought to represent the physical substrate of memory, storing encoded information across distributed brain networks. Consistent with computational theories (Kanerva, 1988), an engram ensemble is sparsely encoded such that not all excitatory neurons are activated during a single memory. This sparsity of the engram is critical for strong and specific memories to be formed (Abatis et al., 2024; Guo et al., 2018; Lesuis et al., 2024). A key question in understanding engram formation is how certain neurons are selectively recruited into these memory circuits while others are not. Compelling evidence indicates that it resides

at least at the cellular level, but with increasing interest for the contribution of the strength of synaptic connections between these activated cells (Brosens et al., 2024; Jung et al., 2024; Kawashima et al., 2009; Lee & Kaang, 2024). This activity-dependent synaptic plasticity in engram neurons is hypothesized to be a substrate for memory and to be both necessary and sufficient for storing information (Martin et al., 2000).

Synaptic plasticity, the ability of synapses to strengthen or weaken in response to activity, underpins the encoding of new memories. Long-term potentiation (LTP), a form of synaptic (or Hebbian) plasticity, has long been considered one of the primary mechanisms through which memories are stored in neural circuits. A second, slower mechanism of synaptic plasticity is that of structural plasticity, which refers to the physical changes in synapses and spines that support the strengthening

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or modification of synaptic connections. AMPA receptors (AMPA), and their dynamic regulation at the synapse, play a central role in synaptic plasticity. These receptors are tetrameric complexes composed of different subunits, which confer distinct functional properties to the receptor. Of particular interest is the GluA1 subunit, which requires LTP stimuli for translocation into and stabilization at synapses (Boehm et al., 2006; Díaz-Alonso et al., 2017; Diering & Hugarir, 2018; Hayashi et al., 2000; Kessels & Malinow, 2009; Shi et al., 2001; Watson et al., 2017). GluA1 germline deletion results in loss of the ability to induce LTP and synaptic plasticity, as well as impaired memory formation (Humeau et al., 2007; Schmitt et al., 2005; Zamanillo et al., 1999). Considering that LTP will more likely take place in memory engram neurons, AMPARs may possibly be of importance in the selection of neurons for memory encoding.

This raises a critical question: How does the presence or absence of GluA1-containing AMPARs influence the recruitment of neurons into memory engrams? We will address this question using global GluA1<sup>KO</sup> mice as well as conditional knockout mice in which GluA1 is depleted in a subset of hippocampal neurons, and evaluate its effects on fear memory learning and engram recruitment. Immediate early genes (IEGs) such as activity regulated cytoskeleton associated protein (Arc) are markers for engram tagging and are used to observe cell (re)activation during memory recall (Denny et al., 2014; Sørensen et al., 2016). Therefore, we employed a mouse model that expresses the fluorescent destabilized protein dVenus under the Arc promoter and crossed this model with GluA1<sup>KO</sup> mice in order to assess memory, engram formation, and synaptic strength in the presence and absence of GluA1. Addressing this question will provide deeper insights into the fundamental processes underlying memory encoding and stability. It may also offer new avenues for therapeutic interventions aimed at enhancing or restoring memory function, especially in conditions where synaptic composition is altered.

## 2. Materials and Methods

### 2.1. Mice

#### 2.1.1. Housing

All mice were housed under standard conditions (20–22 °C, 40–60 % humidity) with littermates (max 4 per cage) on a 12 h/12 h light–dark schedule with standard chow and water available *ad libitum*. Both female and male mice were used. Experiments were performed at the onset of the light phase. All experimental procedures were conducted under the national law and European Union directive 2010/63/EU on animal experiments and were approved by the animal welfare committee of the University of Amsterdam.

#### 2.1.2. Arc::dVenus x GluA1<sup>KO</sup> mice

Homozygous Arc::dVenus reporter mice (gifted by prof. dr. Steven A. Kushner, Erasmus University Rotterdam; Eguchi & Yamaguchi, 2009), expressing the destabilized fluorescent protein dVenus coupled to the Arc promoter, were crossed with homozygous GRIA<sup>-/-</sup> mice (gifted by prof. dr. Richard L. Huganir, John Hopkins University; Kim et al., 2005), to obtain breeding lines heterozygous for Arc::dVenus and with heterozygous knockout of the GluA1 subunit (GluA1<sup>KO</sup>), which were crossbred to obtain Arc::dVenus<sup>HET</sup>-GluA1<sup>KO-HOM</sup> and Arc::dVenus<sup>HET</sup>-GluA1<sup>WT</sup> littermates used for the experiment. All mouse lines were backcrossed for more than 10 generations into a C57BL/6J background derived from Envigo Laboratories (Venray, Netherlands).

#### 2.1.3. GluA1<sup>fl/fl</sup> mice

GluA1<sup>fl/fl</sup> mice (Jackson labs: 019012-B6N129-Gria1<sup>tm2Rsp/J</sup>, RRID: IMSR\_JAX:019012) have a floxed exon 11 allele that allows the deletion of the sequence encoding the GluA1 AMPA receptor subunit in cells expressing Cre, while the floxed exon is still present and expressed at regular levels in the absence of Cre (Fuchs et al., 2007). These mice were

used to locally and temporally knock out the GluA1 subunit of the AMPA receptor in the presence of Cre in the cell. Experimental male and female mice were bred homozygous. This mouse line was backcrossed for more than 10 generations into a C57BL/6J background derived from Envigo Laboratories (Venray, Netherlands).

#### 2.1.4. Virus injections

Mice were pre-treated with buprenorphine (0.1 mg/kg, i.p.) and anesthetized with an isoflurane-oxygen mix (3 % isoflurane for initial induction and 0.8–1.5 % for maintenance). Mice were topically administered lidocaine around the incision site, and holes were drilled bilaterally above the DG (to target AP: −2.0 mm, ML: ±1.3 mm, DV: −1.6 mm relative to bregma) and the CA1 (to target AP: −1.9 mm, ML: ±1.5 mm, DV: −1.4). AAV1.CaMKII-0.4.Cre.SV40 (“AAV-CaMKII-Cre”) (Addgene viral prep # 105558-AAV1; <https://n2t.net/addgene:105558>; RRID:Addgene\_105558) was slowly microinjected (0.4 µl, rate of 0.1 µl/min) via glass micropipettes connected to a microinjector (Nanoject II Auto Injector Kit Drummond Scientific Inc.; 3-000-205A). Following microinjection, micropipettes were maintained in place for an additional 5 min to ensure viral diffusion. Following both microinjections, mice were administered 0.9 % saline (1.0 ml, s.c.) and placed in a clean cage on a heating pad to recover.

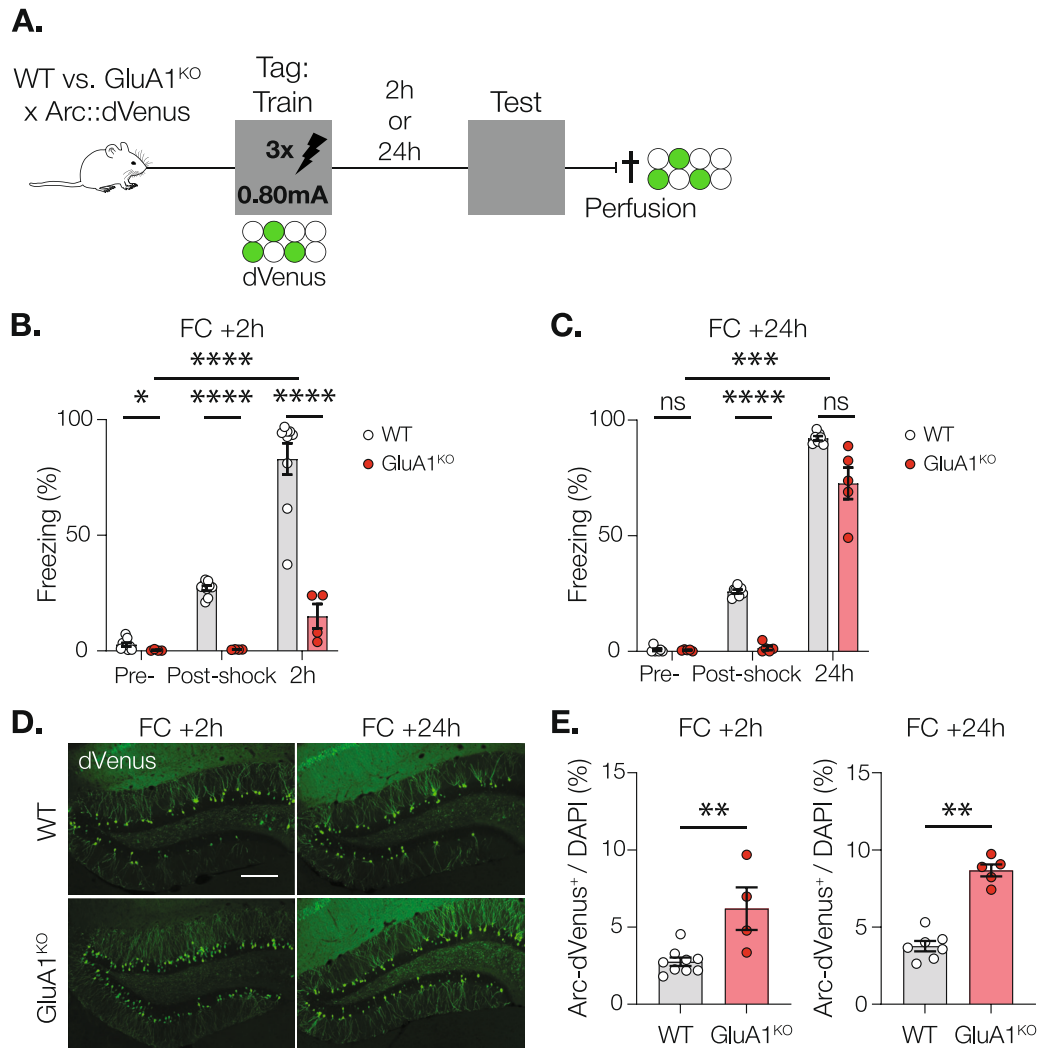
### 2.2. Contextual fear-conditioning

5- to 10-week-old male and female mice were tested in a contextual fear-conditioning paradigm. One week before fear conditioning, mice were housed individually. Mice were put in a soundproof plastic box (W x L x H: 18 x 18.5 x 30 cm) with white noise in the background, white and infrared light, and a stainless-steel grid floor connected to a shock generator. The fear conditioning protocol was conducted using a computer equipped with Ethovision software (version 14.0, Noldus, the Netherlands; RRID:SCR\_000441), and mouse behavior was recorded via a camera (Basler acA1300-30gm GigE, Ahrensburg Germany). Mice were allowed to explore the box for 2 min, after which 3 consecutive 1-sec foot shocks (0.8 mA) were applied with an intershock interval of 60 sec (see Fig. 1A). After the last foot shock, the mice remained in the chamber for 2 min. 2 or 24 h later, the mice were reintroduced in the chamber for 2 min to test their memory for the context. Freezing behavior, i.e., no body movements except related to breathing, was quantified automatically by the Ethovision software.

### 2.3. Tissue preparation

Arc::dVenus x GluA1<sup>KO</sup> or GluA1<sup>WT</sup> mice and GluA1<sup>fl/fl</sup> mice were deeply anesthetized with pentobarbital (120 mg/kg) and transcardially perfused with phosphate-buffered saline (0.1 M PBS, pH: 7.4) and paraformaldehyde (PFA; 4 %), immediately after the fear test or 90 mins after fear conditioning, respectively. The brains were dissected from the skull, postfixed in 4 % PFA for 24 h, and incubated in 30 % sucrose overnight. Frozen brains were sliced into 40 µm coronal sections using a sliding microtome. Slices from Arc::dVenus x GluA1<sup>KO</sup> mice were mounted and coverslipped with Vectashield mounting medium containing DAPI (H-1200, Vector Laboratories Inc.; RRID:AB\_2336790).

Slices from GluA1<sup>fl/fl</sup> mice were first incubated with Fab-fragments (1:250 in PBS, goat anti-mouse IgG Fc-BIOT, SouthernBiotech 1033-08, RRID:AB\_2794333), followed by blocking solution (5 % normal goat serum in PBS with 0.3 % Triton X-100). Slices were washed 3 times with PBS (0.1 M) and incubated with mouse anti-Arc (1:750; Santa Cruz Biotechnology, sc-17839, J3116, RRID:AB\_626696) and rabbit anti-Cre (1:1000; Synaptic Systems, 257 003, RRID:AB\_2619968) overnight on a rotating table at 4 °C. Thereafter, the slices were incubated with Alexa Fluor 568 donkey anti-mouse (1:750; A-10037, Invitrogen) and Alexa Fluor 488 donkey anti-rabbit (1:750, A21206 Invitrogen, RRID:AB\_2535792) in blocking solution. Finally, the slices were washed 3 times with PBS, mounted, and coverslipped with



**Fig. 1.** The GluA1 AMPAR subunit is required for short-term contextual fear memory and sparse Arc expression in the DG. **(A)** Mice were trained in a contextual fear conditioning (CFC) paradigm with 3 times a 1-sec foot shock (0.8 mA). 2 or 24 h later, mice were re-exposed to the context, and immediately thereafter perfused. **(B)** The effects of genotype and trial on freezing behavior. A main effect was found on time ( $F_{1,11} = 63.44$ ,  $p < 0.0001$ ) and genotype ( $F_{1,11} = 64.14$ ,  $p < 0.0001$ ), and an interaction effect was found between trial x genotype ( $F_{2,22} = 28.80$ ,  $p < 0.0001$ ). GluA1<sup>KO</sup> mice showed decreased freezing compared to WT mice pre-shock ( $p = 0.0496$ ), post-shock ( $p < 0.0001$ ) and 2 h post-conditioning ( $p < 0.0001$ ).  $n_{WT} = 9$ ,  $n_{GluA1-KO} = 4$ . **(C)** The effects of genotype and time on freezing behavior 24 h after conditioning. A main effect was found on time ( $F_{1,10} = 620.2$ ,  $p = 0.0001$ ) and genotype ( $F_{1,10} = 65.79$ ,  $p < 0.0001$ ), and an interaction effect was found between trial x genotype ( $F_{2,20} = 13.20$ ,  $p = 0.0002$ ). GluA1<sup>KO</sup> mice showed reduced freezing post-shock ( $p < 0.0001$ ) but were not different from WT pre-shock ( $p = 0.9$ ) or after 24 h ( $p = 0.1$ ).  $n_{WT} = 7$ ,  $n_{GluA1-KO} = 5$ . **(D)** Arc::dVenus fluorescence (green) in the DG of WT (top) and GluA1<sup>KO</sup> mice (bottom) 2 h (left) and 24 h (right) after the contextual fear conditioning. Scale bar: 200 μm. **(E)** The effect of genotype on Arc::dVenus<sup>+</sup> cells / DAPI<sup>+</sup> (%) following CFC. A genotype effect was found 2 h after CFC (Mann-Whitney  $U_{45,46} = 1$ ,  $p = 0.0056$ ), and 24 h after CFC (Mann-Whitney  $U_{28,50} = 0$ ,  $p = 0.0025$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

Vectashield mounting medium with DAPI (H-1200, Vector Laboratories Inc.).

#### 2.4. Quantitative analysis

For the quantification of the number of Arc::dVenus<sup>+</sup> and DAPI<sup>+</sup> neurons in DG and CA1, slices were imaged at 10x using a Nikon DS-Ri2 microscope. Arc::dVenus fluorescence was imaged using a 510 nm excitation wavelength and 460 nm for DAPI. Arc::dVenus<sup>+</sup> and DAPI<sup>+</sup> neurons were manually counted by an experimenter blinded from their genotype using Fiji (ImageJ) graphic software (v.2.0.0-rc-65/1.5w, National Institutes of Health, RRID: SCR\_002285). The Arc::dVenus<sup>+</sup> fraction was quantified as a percentage of the DAPI<sup>+</sup> cell density in the granule layer of the DG and in the CA1 in 8 series bilaterally (bregma

−1.34 mm to −2.54 mm) per animal.

To analyze the relative expression of Arc<sup>+</sup> neurons within the GluA1-containing and GluA1-lacking Cre<sup>+</sup> vs. Cre<sup>-</sup> neuronal population, brain areas were thresholded and binarized, and individual neurons were classified as either Cre<sup>+</sup> or Cre<sup>-</sup>. Next, thresholded and binarized images of Arc stainings were overlaid on these images, and neurons were classified into one of the following categories: Cre<sup>-</sup>/Arc<sup>-</sup>, Cre<sup>+</sup>/Arc<sup>-</sup>, Cre<sup>-</sup>/Arc<sup>+</sup> or Cre<sup>+</sup>/Arc<sup>+</sup>.

#### 2.5. Whole-cell electrophysiology

Acute hippocampal slices were prepared from 3- to 5-week-old mice. Dissection was done in ice-cold sucrose cutting solution containing (in mM): 2.5 KCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 D-glucose, 230 Sucrose,

0.5 CaCl<sub>2</sub>, 10 MgSO<sub>4</sub>, bubbled with 95 % O<sub>2</sub>/ 5 % CO<sub>2</sub>. Brain slices (400 μm) were cut using a vibratome (Thermo Scientific) and placed in a holding chamber containing ACSF supplemented with (in mM) 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 20 glucose, and bubbled with 95 % O<sub>2</sub>/ 5 % CO<sub>2</sub>. They were allowed to recover at 34 °C for 40 min then at room temperature for at least 40 min.

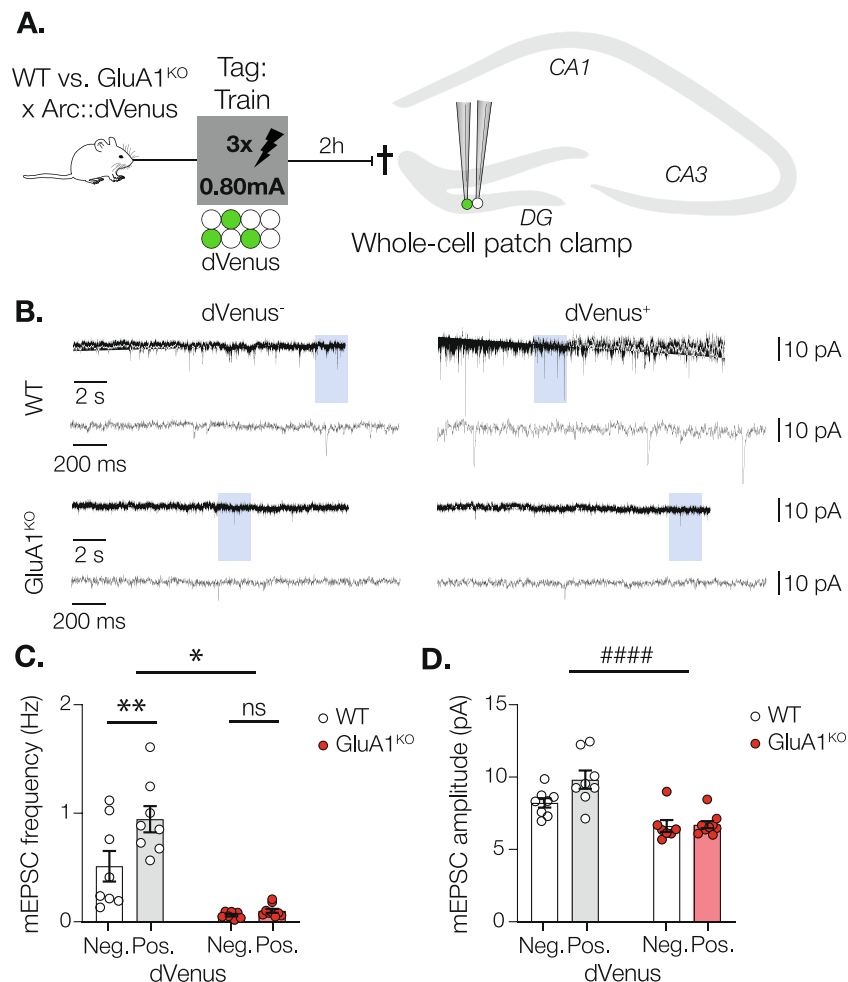
Whole-cell recordings (3–5 MΩ pipettes, R<sub>access</sub> < 26 MΩ, and R<sub>input</sub> > 10 x R<sub>access</sub>) were made at –60 mV in ACSF containing TTX (1 μM) and picrotoxin (50 μM) at 28 °C. Patch recording pipettes were filled with an internal solution containing 115 mM CsMeSO<sub>3</sub>, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl<sub>2</sub>, 4 mM Na<sub>2</sub>-ATP, 0.4 mM Na-GTP, 10 mM sodium phosphocreatine, and 0.6 mM EGTA, at pH 7.25. Data was acquired using a Multiclamp 700B amplifier (Molecular Devices). Miniature excitatory postsynaptic potential (mEPSC) data are based on at least 100 events or 5 min of recording. Data were analyzed with MiniAnalysis (Synaptosoft). Individual events above a 5 pA threshold were manually selected.

## 2.6. Statistical analysis

Statistical tests were performed using Prism 9 (GraphPad Software, LLC, San Diego, CA, RRID: SCR\_014284). A two-way ANOVA followed by a Šidák multiple comparisons test was performed for multiple group analysis. Mann-Whitney U was used to compare groups at a fixed time. All values are reported as mean ± standard error. The null hypothesis was rejected upon a significance level of 0.05.

## 3. Results

We set out to investigate the role of the AMPA receptor (AMPA) subunit GluA1 in fear memory processing (Fig. 1A-C). We used mice with global germline knockout of the GluA1 subunit of the AMPA receptor (GluA1<sup>KO</sup>) and wild-type (WT) littermates. All mice were also Arc::dVenus-positive, expressing a destabilized Venus fluorescent protein (dVenus) under the control of the Arc promoter, which enables the identification of recent experience-dependent activation of neurons (Eguchi & Yamaguchi, 2009; Rao-Ruiz et al., 2019). Arc::dVenus transgenic mice continue to express dVenus in the dentate gyrus of the



**Fig. 2.** GluA1<sup>KO</sup> mice have impaired synaptic strength and no Arc::dVenus-specific strengthening of the synapse. **(A)** Mice were trained in a contextual fear conditioning (CFC) paradigm with 3 times a 1-sec foot shock (0.8 mA), and after 2 h, hippocampal slices were prepared for patching of Arc::dVenus<sup>+</sup> and Arc::dVenus<sup>-</sup> neurons. **(B)** Typical miniature excitatory postsynaptic current (mEPSC) of Arc::dVenus<sup>-</sup> (left) and Arc::dVenus<sup>+</sup> (right) cells of WT (top) and GluA1<sup>KO</sup> (bottom) mice. **(C)** The effects of genotype and Arc::dVenus expression on mEPSC frequency. A main effect was found for genotype ( $F_{1,28} = 47.63$ ,  $p < 0.0001$ ) and dVenus ( $F_{1,28} = 6,366$ ,  $p = 0.0176$ ), and an interaction effect was found between genotype x Arc::dVenus ( $F_{1,28} = 4.369$ ,  $p = 0.0458$ ). WT mice showed a significantly higher frequency in Arc::dVenus<sup>+</sup> neurons compared to Arc::dVenus<sup>-</sup> neurons ( $p = 0.0056$ ), while this was not different in GluA1<sup>KO</sup> mice ( $p = 0.94$ ). **(D)** The effects of genotype and Arc::dVenus expression on mEPSC amplitude. A main effect was found on genotype ( $F_{1,28} = 31.06$ ,  $p < 0.0001$ ), but not on Arc::dVenus ( $F_{1,28} = 4.034$ ,  $p = 0.0543$ ), and no interaction effect between genotype x Arc::dVenus was found ( $F_{1,28} = 3.201$ ,  $p = 0.0844$ ).  $n_{WT\ dVenus^-} = 8$ ,  $n_{WT\ dVenus^+} = 7$ ,  $n_{GluA1-KO\ dVenus^-} = 8$ ,  $n_{GluA1-KO\ dVenus^+} = 7$ ; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ; genotype effect: ####  $p < 0.0001$ .

hippocampus following fear conditioning over a 24-hour time span, thus reflecting neurons activated during the learning experience at this time point (Rao-Ruiz et al., 2019).

We exposed mice to a contextual fear conditioning paradigm consisting of three 0.8 mA electric foot shocks (Fig. 1A). Whereas WT mice display a freezing response immediately following the foot shocks, such post-shock freezing was absent for GluA1<sup>KO</sup> mice (Fig. 1B-C). When re-exposed to the fear context 2 h after conditioning, GluA1<sup>KO</sup> mice showed a minor freezing response that was significantly lower than in WT mice (Fig. 1B). In comparison, freezing was not different between the groups at 24 h (Fig. 1C). These data indicate that short-term memory is fully dependent on the expression of GluA1, while 24 h memory is largely intact in GluA1<sup>KO</sup> mice. Next, we investigated how the absence of the GluA1 AMPAR subunit affected Arc expression, suggestive of engram neurons. The Arc::dVenus<sup>+</sup> fraction of cells in the DG was significantly higher in the GluA1<sup>KO</sup> mice compared with WT littermates at both 2 h and 24 h after fear conditioning (Fig. 1D-E). Thus, GluA1<sup>KO</sup> mice express Arc in a higher fraction of neurons in the DG after fear learning. To investigate whether this increased number of Arc-positive neurons is also seen under basal conditions, we analyzed Arc expression in WT and GluA1<sup>KO</sup> mice without fear conditioning (supplemental fig. 1A). GluA1<sup>KO</sup> mice showed a significantly higher fraction of Arc<sup>+</sup> neurons, indicating an increased likelihood of a neuron to become Arc<sup>+</sup> in the absence of GluA1 (supplemental fig. 1B-1C).

To investigate the role of the GluA1 AMPAR subunit on synaptic strength onto DG neurons after fear conditioning, we performed whole-cell electrophysiology of Arc::dVenus<sup>+</sup> and Arc::dVenus<sup>-</sup> DG neurons 2 h after conditioning (Fig. 2A). In WT mice, synapses were on average stronger in Arc::dVenus<sup>+</sup> DG neurons than in Arc::dVenus<sup>-</sup> neurons, as reflected by an increased mEPSC frequency (Fig. 2B-2C). In comparison to WT mice, GluA1<sup>KO</sup> mice showed a substantially lower frequency of mEPSC events, which have, on average, a lower amplitude, with no difference between Arc::dVenus<sup>-</sup> and Arc::dVenus<sup>+</sup> neurons (Fig. 2B-2D). Therefore, Arc<sup>+</sup> neurons show more synaptic potentiation than neurons that do not express Arc after a fearful experience in WT but not GluA1<sup>KO</sup> mice. These data indicate that this is a GluA1-dependent process.

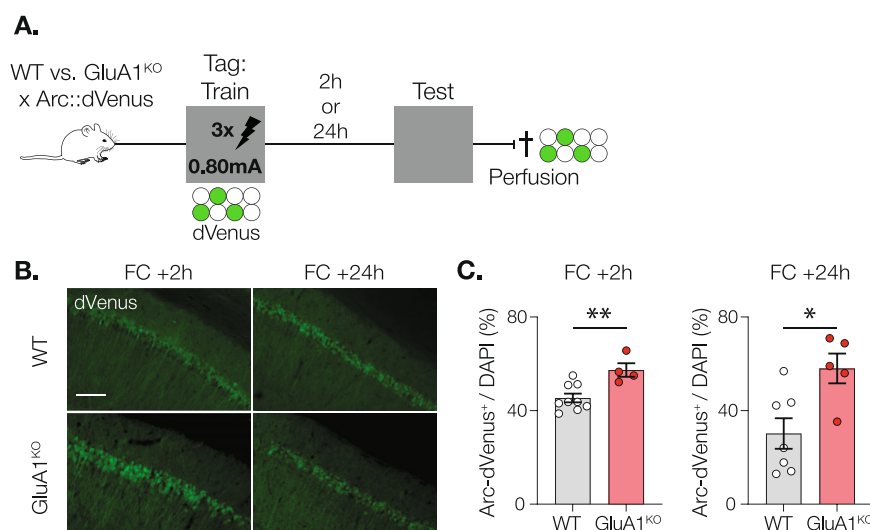
Whereas memory formation in the DG is characterized by sparse, strong activation of a small subset of neurons, engram formation in the CA1 is more widely distributed among neurons (Ramirez et al., 2013).

We investigated fear memory encoding in the CA1 to determine whether the effects of GluA1 on neuronal Arc expression are DG-specific (Fig. 3A). Compared with their WT littermates, GluA1<sup>KO</sup> mice had a higher fraction of Arc::dVenus<sup>+</sup> neurons in the dorsal CA1 of the hippocampus after fear conditioning (Fig. 3B-C). Therefore, the changes in Arc expression as a consequence of a lack of GluA1 expression are not isolated to the DG.

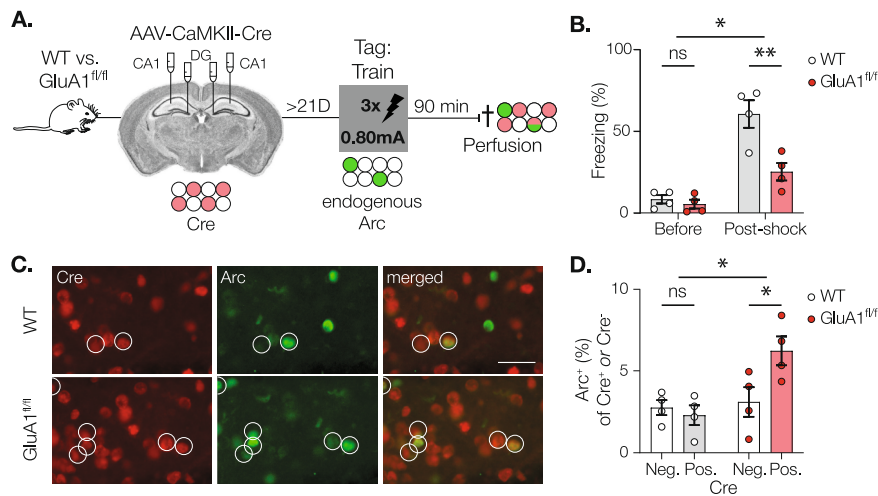
GluA1<sup>KO</sup> mice lack expression of the GluA1 subunit throughout the brain, including during development. To investigate whether the absence of the GluA1 AMPAR subunit is driving the changes in Arc expression, we knocked down the GluA1 AMPAR subunit in approximately 50 % of CA1 and DG excitatory neurons by injecting a low titer AAV-CaMKII-Cre in GluA1<sup>fl/fl</sup> mice, to obtain a local and temporally constraint knockout of GluA1 in the hippocampus (Fig. 4A). The local knockout resulted in decreased post-shock freezing levels (Fig. 4B), similar to what we observed with the global GluA1<sup>KO</sup> mice (Fig. 1B-C). In the GluA1<sup>fl/fl</sup> mice, the portion of Arc<sup>+</sup> cells was higher in the Cre-infected neurons (Cre<sup>+</sup>) than the non-infected neurons (Cre<sup>-</sup>), while this effect was absent in WT mice. I.e., knocking out the GluA1 AMPAR subunit increased the chance of a neuron becoming Arc<sup>+</sup> (Fig. 4C-D). These results confirm our initial observation that the GluA1 subunit is required for sparse expression of Arc, and that in the absence of GluA1 subunits, the number of Arc<sup>+</sup> neurons increases. Altogether, these data point towards a critical role of the GluA1 AMPAR subunit in sparse memory engram encoding.

#### 4. Discussion

In this study, we investigated the role of the AMPAR subunit GluA1 on contextual fear memory encoding. We report that global knockout of GluA1 results in minimal freezing immediately following the foot shock and 2 h after training, but mice display substantial freezing at 24 h, indicative of intact 24 h contextual fear memory expression. While we observed this deficit in short-term memory, local knockout of the GluA1 subunit also resulted in increased expression of the immediate early gene Arc in the dentate gyrus and CA1 neurons of the dorsal hippocampus, suggesting a larger engram in GluA1 knockout mice. Interestingly, depleting the GluA1 subunit in a subset of excitatory neurons in the DG and CA1 increased the chance of neurons without GluA1 becoming Arc<sup>+</sup>. Altogether, these observations suggest that GluA1 might



**Fig. 3.** GluA1 AMPAR subunits are also required for sparse Arc expression in the CA1. (A) Mice were trained in a contextual fear conditioning (CFC) paradigm as described in Fig. 1A. (B) Arc::dVenus antibody staining (green) in the CA1 of WT (top) and GluA1<sup>KO</sup> mice (bottom) 2 h (left) and 24 h (right) after the contextual fear conditioning. Scale bar: 200  $\mu$ m. (C) The effect of genotype on Arc::dVenus<sup>+</sup> cells / DAPI<sup>+</sup> following CFC. A genotype effect was found 2 h after CFC (Mann-Whitney  $U_{46.5, 44.5} = 1.5$ ,  $p = 0.0084$ ,  $n_{WT} = 9$ ,  $n_{GluA1-KO} = 4$ ), and 24 h after CFC (Mann-Whitney  $U_{32.46} = 4$ ,  $p = 0.0303$ ,  $n_{WT} = 7$ ,  $n_{GluA1-KO} = 5$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .



**Fig. 4.** Local GluA1 knockout in the dorsal DG and CA1 increases the likelihood of a neuron expressing Arc. **(A)** An AAV-CaMKII-Cre virus was injected in the dorsal DG and CA1 of WT and  $GluA1^{fl/fl}$  mice. > 21 days after the virus injection, the mice were trained in a contextual fear conditioning paradigm (CFC) as described in Fig. 1A, perfused 90 min later, and stained for Cre and Arc expression. **(B)** The effect of genotype and foot shock on freezing behavior. A main effect was found on the foot shock ( $F_{1,6} = 61.54$ ,  $p = 0.0002$ ) and genotype ( $F_{1,6} = 10.36$ ,  $p = 0.0182$ ), and an interaction effect was found for genotype x foot shock ( $F_{1,6} = 12.44$ ,  $p = 0.0124$ ).  $GluA1^{fl/fl}$  mice showed decreased freezing compared to WT mice after the foot shock ( $p = 0.001$ ) but not before the foot shock ( $p = 0.91$ ). **(C)** Cre (red) and Arc (green) antibody staining in the DG of WT (top) and  $GluA1^{fl/fl}$  mice (bottom) after contextual fear conditioning. White circles indicate colocalization between  $Cre^+$  and  $Arc^+$  neurons. Scale bar: 10  $\mu m$ . **(D)** The effect of genotype and virus expression on the fraction of  $Arc^+$  neurons within the  $Cre^-$  or  $Cre^+$  population. A main effect was found on genotype ( $F_{1,6} = 7.792$ ,  $p = 0.0315$ ), but not on virus ( $F_{1,6} = 3.548$ ,  $p = 0.1086$ ), and an interaction effect for virus x genotype ( $F_{1,6} = 6.470$ ,  $p = 0.0439$ ). A significantly larger fraction of the  $Cre^+$  portion of the DG was  $Arc^+$  than of the  $Cre^-$  portion in  $GluA1^{fl/fl}$  mice ( $p = 0.0402$ ), but not in WT mice ( $p = 0.88$ ).  $n_{WT-Arc^-} = 4$ ,  $n_{WT-Arc^+} = 4$ ,  $n_{GluA1^{fl/fl}-Arc^-} = 4$ ,  $n_{GluA1^{fl/fl}-Arc^+} = 4$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

play a critical role in the sparse (vs. dense) encoding of the engram.

#### 4.1. *GluA1*-containing AMPARs constrain the number of *Arc*<sup>+</sup> neurons

IEGs such as *Arc* are the target genes of CREB and are upregulated by neuronal activation (Kawashima et al., 2009; Sheng et al., 1991), and those neurons that have increased CREB levels are therefore considered to be highly likely to become part of the memory engram (Han et al., 2007). As such, neurons with high *Arc* expression are considered a proxy for engram neurons, and it is thought that the expression of these IEGs is related to the mechanism by which engrams are formed (Miyashita et al., 2018). We demonstrate that the likelihood of a neuron expressing *Arc* was increased if that neuron lacked *GluA1*. This is in line with a previous study, which showed that AMPA receptor activity in cultured neurons and organotypic slices inhibits the transcription of *Arc* through a G-protein-dependent signaling pathway (Korb & Finkbeiner, 2011; Rao et al., 2006). We here demonstrate that such an AMPAR-driven inhibition of *Arc* expression also occurs *in vivo*. Specifically, global *GluA1* knockout mice expressed higher levels of *Arc* following fear learning, both shortly following the learning event (when fear memory was still impaired), as well as 24 h later when fear memory was retained. Indeed, knockout of *GluA1* only in a subset of excitatory neurons in the hippocampus demonstrated that those neurons without *GluA1* were more likely to express *Arc* than those neurons that had endogenous *GluA1* expression, and thus supports our observation that fewer *GluA1* AMPARs in a neuron result in increased *Arc* expression.

#### 4.2. *GluA1*-containing AMPARs are critical for short-term memory expression

*GluA1*-containing AMPARs are rapidly inserted into synapses upon LTP induction and upon a learning experience (Hayashi et al., 2000; Kessels & Malinow, 2009; Makino & Malinow, 2009; Rumpel et al., 2005; Shi et al., 2001). *GluA1*-dependent synaptic potentiation at CA1 neurons is critically implicated in the formation of contextual fear memories (Mitsushima et al., 2011). In mice lacking *GluA1* subunits, associative LTP is absent at CA3 to CA1 synapses (Zamanillo et al.,

1999), and AMPAR currents at CA1 synapses are substantially reduced (Lu et al., 2009; Renner et al., 2017). At synapses onto DG neurons, LTP induction leads to the rapid synaptic insertion of *GluA1* subunits during the early phase of LTP (Williams et al., 2007). Indeed, we observed that following learning, synaptic AMPAR currents at DG *Arc*:dVenus<sup>+</sup> neurons are strengthened in wild-type (WT) mice, as previously reported (Lesuis et al., 2021), but not in  $GluA1^{KO}$  mice. Moreover, synaptic strength in DG neurons of  $GluA1^{KO}$  mice is substantially reduced, irrespective of whether these neurons express *Arc*. Interestingly, synapse strength appears to be more dependent on *GluA1* expression for DG neurons when we compare our mEPSC recordings with those in other brain regions such as from CA1 neurons (Lu et al., 2009; Renner et al., 2017), lateral amygdala neurons (Humeau et al., 2007) or cerebellar Purkinje neurons (Gutierrez-Castellanos et al., 2017), suggesting that DG neurons express more *GluA1* relative to other subunits and/or experience relatively more *GluA1*-dependent LTP compared with these other types of neurons.

Interestingly, our observations indicate that  $GluA1^{KO}$  mice exhibited substantially reduced freezing on the short-term (2 h post-conditioning), while these mice showed comparable freezing levels to WT controls at the 24 h memory test. Possibly, WT mice display a ceiling effect in our experiment, since they showed freezing levels at 24 h close to 100 % as a consequence of using a strong conditioning protocol (3 shocks of 0.8 mA; Fig. 1B). Indeed, it was previously shown that with a milder protocol (3 shocks of 0.4 mA) long-term contextual fear memories continue to be impaired in  $GluA1^{KO}$  mice (Humeau et al., 2007). Thus, *GluA1*-dependent plasticity is critical for short-term memories, but might also contribute to long-term memories. This aligns with previous findings linking *GluA1*-containing AMPARs to rapid synaptic potentiation following high-frequency stimulation and LTP induction (Boehm et al., 2006; Diering & Huganir, 2018). We speculate that *GluA1*-independent forms of synaptic plasticity that require protein synthesis, such as for instance NMDAR-plasticity, *GluA2/3*-plasticity, and structural plasticity (Renner et al., 2017; Williams et al., 2007), account for long-term but not short-term memories, therefore possibly playing a role in memory consolidation at the synapse level. The production of *Arc* may also contribute to the synaptic consolidation of memories, considering that

Arc-knockout mice display intact short-term memory but impaired long-term memory (Plath et al., 2006). The notion that GluA1<sup>KO</sup> mice have a selective short-term or working memory deficit is further implicated by their poor performance in single-trial tests for spatial memory (Humeau et al., 2007; Sanderson et al., 2007), but good performance in multiple-trial tests for spatial reference memory (Inta et al., 2014; Sanderson et al., 2007; Zamanillo et al., 1999). We note that inhibition of CaMKII, a protein that drives GluA1-containing AMPARs into synapses during LTP (Hayashi et al., 2000), also resulted in an impairment of short-term but not long-term memory (Shin et al., 2025), similar to what we observed in GluA1<sup>KO</sup> mice. These findings indicate that GluA1-mediated plasticity is critical for short-term memory formation, and is only partly responsible for the expression of long-term memory. Further studies into the effects of local GluA1 knockout could shed light on the spatial contribution of different brain regions on this discrepancy between short- and long-term memory expression in the absence of GluA1.

#### 4.3. GluA1 is necessary for sparse engram formation

The overall size and sparsity of an engram ensemble are remarkably constant across types of memory (Gouty-Colomer et al., 2016; Hsiang et al., 2014; Zhang et al., 2020). The increase in Arc<sup>+</sup> neurons following local and global GluA1 deletion suggests that GluA1 contributes to achieving sparse memory encoding by restraining excessive recruitment of neurons into engrams. The selective, sparse incorporation of neurons into memory circuits is thought to underlie the specificity and efficiency of memory storage, and a shift toward a larger engram has been associated with less precise, more generalized encoding of memories (Lesuis et al., 2024). Likewise, this altered encoding may potentially affect other properties of memory retrieval, such as the efficiency with which a memory can be extinguished. Sparse memory representation is theorized to prevent overlap between memory traces, reducing interference and enhancing memory retrieval accuracy (Kanerva, 1988; Lesuis et al., 2021, 2024). By regulating excitatory drive through GluA1-containing AMPARs, the brain may fine-tune which neurons participate in engram circuits, optimizing both the strength and precision of memory representations. Additionally, the increased Arc::dVenus levels under baseline conditions suggest that neuronal allocation may also be affected. This implies that changes in neuronal properties prior to stimulus presentation could render specific neurons more or less likely to be selected for inclusion in the engram. Such pre-stimulus changes could further influence how sparse or distinct memory representations are formed, potentially impacting memory encoding, retrieval, and resistance to interference. Our data from global and local GluA1 deficient animals might suggest that under the artificial conditions of total absence of GluA1 in the synapse, and despite increased IEG levels, (short-term) memory formation is hampered. This either means that the observed IEG<sup>+</sup> neurons constitute noise, or that the synaptic connectivity of these neurons in the microcircuitry and/or macroconnectivity of the brain need to be strengthened before these activated neurons become proper engram neurons that encode a memory. Further hallmark loss-of-function, gain-of-function, and mimicry experiments, that have all been done to demonstrate the critical role of engrams in memory (Denny et al., 2014; Josselyn & Tonegawa, 2020; Vetere et al., 2019) will need to be carried out to further investigate the hypothesis that the GluA1 subunit is necessary for sparse engram formation.

#### 4.4. Conclusion

Understanding the molecular mechanisms that determine engram neuron selection has broader implications for our understanding of memory formation and storage. In neurodegenerative diseases such as Alzheimer's disease, disruptions in synaptic composition, including reductions in AMPAR levels at synapses (Hsieh et al., 2006; Reinders et al., 2016), may impair the formation of stable memory engrams, contributing to the cognitive deficits seen in these conditions. Moreover, the

role of GluA1 in synaptic plasticity raises important questions about the mechanism behind how a neuron is aware it has no or fewer GluA1 AMPARs in its synapses, and thus influences engram formation. Addressing these questions will provide deeper insights into the fundamental processes underlying memory and may offer new avenues for therapeutic interventions aimed at enhancing or restoring memory function.

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#### CRedit authorship contribution statement

**Thijse S. Willems:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hui Xiong:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Helmut W. Kessels:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Sylvie L. Lesuis:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nlm.2025.108031>.

#### Data availability

Data will be made available on request.

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