Cerebellin-4 (Cbln-4) signalling at synapses mediating memory-guided behaviours

Master's Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

Vachan S J

20191059

Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

Date: April, 2024

Supervisor: Jai Santosh Polepalli

From May 2023 to Mar 2024

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled **Cerebellin-4 (Cbln-4) signalling mediating memory-guided behaviours** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune, represents study/work carried out by **Vachan S J**, at the National University of Singapore, under the supervision of **Dr Jai Santosh Polepalli**, Assoc. Professor, Department of Anatomy, during the academic year 2023-2024.

Jai Santosh Polepalli

Committee:

Dr. Jai Santosh Polepalli

Dr. Raghav Rajan

This thesis is dedicated to all the journeys

Declaration

I hereby declare that the matter embodied in the report entitled **Cerebellin-4 (Cbln-4) signalling mediating memory-guided behaviours** are the results of the work carried out by me at the Department of Anatomy, National University of Singapore, under the supervision of Dr. Jai Santosh Polepalli, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Vachan S J

20191059

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Abstract

Psychiatric and neurodegenerative disorders present with distinct and wide variety of symptoms. Learning and memory impairment is one common cognitive manifestation among these. However, at the molecular and circuitry levels, our knowledge of the neural operations and pathology behind these symptoms is restricted. In this study, we show that presynaptic signalling of Cerebellin4 (Cbln4), a synaptic protein, to the dentate gyrus (DG), are essential in mediating pattern separation ability in mice. This suggests that Cbln4 plays a vital role in encoding and recall of precise memory, via presynaptic signalling to the DG. This disability of Cbln4-mediated pattern separation is interestingly paired with an increased rate of neurogenesis in the DG. We also find that the deletion of Cbln4 in the DG resulted in a reduced frequency of miniature inhibitory post-synaptic currents (mIPSCs) in the dentate gyrus granule cells. However, learning and memory skills tested by a contextual fear conditioning and extinction task remain unimpaired. Altogether, Cbln4 signalling from presynaptic inputs to the DG and within plays a crucial role in preserving the molecular and circuit level accuracy of the memory code, with an experience-dependent modulation of neurogenesis.

Acknowledgments

I would like to express my deep gratitude to Dr Jai Polepalli for his valuable guidance, and support throughout the project and for giving me this chance to study under him. I would like to thank my TAC member, Dr. Raghav Rajan for following up on my project and for his valuable advice. I would also like to thank Dr. Aurnab Ghose for giving me the opportunity to work in his lab and helping me grow as a young researcher.

I am thankful to Abhishek Rale, Niranjan Biju and Usashi Guha Roy for teaching me the various techniques when I first started at AG lab. I would like to thank the CeLS animal vivarium staff for their assistance. I am thankful to my lab members at the Polepalli lab, especially Joanne Koh and Louise Goh-Zi Ning for teaching me the very many techniques and their valuable insight. I want to thank the NUS AIL microscopy facility and Phenotype core of the CeLS vivarium. I would like to thank the Department of Anatomy - Yong Loo Lin school of medicine, Institute of Life Science Neurobiology Program and the Healthy Longevity Translational Program for the great facility and faculty. I would like to thank Singapore Ministry of Education for the financial support and Healthy Longevity Translational Program for the monthly stipend.

I would like to thank all my friends (Chapatis), Drishti, Shashank, Akshata, Daksh, Kaustubh, Theja and many others who supported me and helped me through the 5-year journey. I am grateful to Shruti, Chaitanya and Archana for their friendship over the course of this project. I would also like to thank the IISER community for giving me some of the best few years of my life, and to everyone who was involved in the IISM football and athletics team over the many sports meets. A special thanks to the Black Dogs FC, forever champions and the best team anyone could ask for.

I would like to thank my parents for their support and presence throughout my life. A special thanks to Aditi for making my time in Singapore memorable and for her support and company.

Contributions

Contributor name	Contributor role
JSP	Conceptualization Ideas
JSP	Methodology
VSJ	Software
VSJ	Validation
VSJ	Formal analysis
VSJ	Investigation
JSP and VSJ	Resources
VSJ	Data Curation
VSJ	Writing - original draft preparation
JSP and VSJ	Writing - review and editing
VSJ	Visualization
JSP	Supervision
JSP	Project administration
JSP	Funding acquisition

Chapter 1 Introduction

1.1 Psychiatric Disorders

Psychiatric disorders are a growing global concern. Some of these disorders include autism spectrum disorder (ASD), schizophrenia, and ADD (Attention Deficit Disorder). Although classified as distinct disorders, they often share overlapping risk factors, in addition to the overlap in behavioural symptoms that individuals with these disorders present with. Genetic studies consistently reveal that both common and rare genetic variants contribute to the increased risk of psychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). Some of the abnormalities that are found to be common to these disorders are deficits in executive functions, emotional control, learning and memory (Pittenger and Duman, 2008; Millan et al., 2012).

This thesis will investigate the underlying molecular, synaptic, cellular and circuit mechanisms of abnormal learning and memory often seen in psychiatric disorders, as well as the common defects observed in these cognitive functions. These abnormalities affect an individual's ability to acquire, store and retrieve information, significantly affecting their quality of life (Stępnicki et al., 2018). Yet, there is no medical intervention to date, to ameliorate these abnormalities in individuals suffering from its consequences. For example, in schizophrenia, symptoms are classified into three groups: positive (hallucinations and thought disorders which form the care of the disorder), adverse (social withdrawal and flat effect), and cognitive (learning and attention disorders). Existing treatments for schizophrenia are efficient for about half the patients and primarily relieve only the positive symptoms (Stępnicki et al., 2018). The causes and mechanisms of schizophrenia, among other psychiatric disorders, remain elusive despite ongoing research efforts. While psychiatric disorders are multifaceted, we can gain valuable insight into the neurobiological mechanisms that contribute to these disorders by characterising their genotypic and phenotypic commonalities.

Psychiatric disorders have a strong genetic component associated with them (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013); mutations, deletions, and copy number variations in multiple genes have been reported to participate in the aetiology of these disorders (Andreassen et al., 2023). A vast majority of gene abnormalities that present as psychiatric disorders encode proteins that are associated with synapses (Brainstorm Consortium et al., 2018). However, the

role of these genes in synaptic function and in disease aetiology is not well understood.

1.2 Synapse as a unit of computation in learning and memory, and loci for disease

Synapses are special junctions that enable neuronal communication through synaptic transmission. These structures comprise a presynaptic terminal that contains vesicles containing neurotransmitters and molecular machinery that facilitates neurotransmitter release and a postsynaptic terminal which contains receptors that bind the released neurotransmitters and molecular machinery that facilitates receptor anchoring, trafficking and downstream signalling from the synapse. Synapses are plastic and undergo experience-dependent changes in structural and functional properties (Kolb and Gibb, 2011), such that an individual synapse forms a basic unit of computation in the brain, and changes in the properties of these synapses in an experience-dependent manner (synaptic plasticity) mediates various adaptations including learning and memory that are crucial for the survival of an animal.

At the cellular level, excitatory neurons form the building blocks of memory formation. This is achieved by regulating their synaptic strengths which confers the ability to hold information at the synapse level. Excitatory neurons' postsynaptic membranes contain two distinct types of ligand-gated channels serving as receptors for glutamate, the neurotransmitter for excitatory neurons - AMPARs (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptor) and NMDARs (N-methyl-D-aspartate receptors). Upon glutamate binding, AMPARs generate a small and rapid excitatory postsynaptic potential by facilitating the influx of potassium and sodium ions. In contrast, the second class, NMDARs, are associated with Hebbian behavior observed in synapses (Mayer et al., 1984). Activation of NMDARs permits the influx of calcium, potassium, and sodium ions only when both the following conditions are met: glutamate is bound, and the synaptic membrane experiences strong depolarization, with a pronounced correlation between these events.

Long-term potentiation (LTP) - “long-lasting increase in synaptic strength”, is a form of synaptic plasticity essential for the formation of memories. LTP is induced when action potentials are fired repeatedly in the presynaptic terminal, which results in a release of glutamate, which, in turn, causes the postsynaptic neurons also to fire an action potential. The Hebbian postulate states that neurons that fire together, wire together. This governs the direction and strength of synaptic plasticity, and can be used to explain this phenomena. Long-term depression (LTD) is defined as the

persistent decrease in synaptic strength accompanied by a reduction in the magnitude of AMPARs (Goto, 2022).

1.3 Association of Learning and Memory with Synaptic Cell Adhesion Molecules (CAMs)

Synaptic cell-adhesion molecules (CAMs) are cell surface molecules that link the presynaptic terminal to the postsynaptic terminal. Synaptic CAMs have demonstrated trans interactions to produce synapses (Frei and Stoeckli, 2014). Synaptic CAMs are integral parts of synapses and are shown to have critical roles in maintaining the synaptic structure during brain development (de Agustín-Durán et al., 2021) and through adulthood (Fields and Itoh, 1996). There is also growing evidence of synaptic CAMs' involvement in regulating synaptic plasticity, and they are recognised for playing a crucial role in initiating the initial points where synapses form and enable the subsequent interaction of different molecular elements during synaptogenesis (Washbourne et al. 2004). This intricate process establishes the specificity of synapse types, emphasising the fundamental role CAMs play in establishing neural connectivity (Kasem et al., 2018; Washbourne et al., 2004).

Based on genome-wide association studies, numerous psychiatric diseases which share a common impairment in learning and memory also have similar molecular defects (Cuttler et al., 2021; Brainstorm Consortium et al., 2018). Neurexins and neuroligins, a family of synaptic CAMs which act as synaptic organisers in mammals, were implicated in these processes.

Specifically, alterations in genes encoding the synaptic CAMs, neurexins (Nrxn) and neurexin interacting proteins have been incriminated in a number of psychiatric disorders - schizophrenia (Ivorra et al., 2014; Mirnics et al., 2000) and ASD (Sanders et al., 2015; Camacho-Garcia et al., 2012), Bipolar Disorder (Kuo et al., 2014).

Therefore, it is likely that mutations in synaptic CAM families could manifest as phenotype deficits such as learning and memory.

1.4 Cerebellins

Cerebellins are a family of neurexin-interacting proteins. These are secreted adaptor proteins and one of their main functions is to connect postsynaptic receptors and the presynaptic neurexins (Cbln 1-3 bind GluD1/2 while Cbln4 binds deleted in colorectal cancer (DCC) and Neogenin-1). Cblns are implicated in neuropsychiatric disorders for

deficits in cognition. Vertebrates produce four homologous Cblns, and are described as “small secreted proteins composed of an N-terminal cysteine-rich sequence and a C-terminal C1q-domain. Cerebellin C1q domains assemble into trimers, while their N-terminal cysteine-rich sequence dimerises the C1q-domain trimers into hexamers” (S. J. Lee et al., 2012). Although Cbln3 requires Cbln1 for proper assembly and function, the other Cblns can do so autonomously (D. Bao et al., 2006). Cblns are classified as synaptic CAMs that facilitate trans-synaptic interactions and the function and fomation of synapses (Ito-Ishida et al., 2008).

Cbln1 and 2 bind to presynaptic neurexins with strong affinity (Matsuda and Yuzaki, 2011), while Cbln4 binds with a lower affinity (Wei et al., 2012). Postsynaptically, GluD1 and GluD2 bind to neurexin-bound Cbln1 and Cbln2. GluDs are two related receptors in the family of ionotropic glutamate receptors (iGluRs), but do not seem to function like glutamate receptors (Dai et al., 2021) and DCC and Neogenin-1 are receptors that mediate axon guidance in the developing brain.. Neurexin-bound Cbln4 binds to neogenin-1 and DCC with high affinity, but not with GluDs (Zhong et al., 2017). Interestingly, Cbln4 was found to function via interactions with postsynaptic GluD1 in the somatosensory cortex (Fossati et al., 2019), although biophysical investigations show that Cbln4 fails to bind GluD1 (Zhong et al., 2017; Yasumura et al., 2012).

Throughout the brain, Cbln1, 2, and 4 are secreted sporadically. On the other hand, Cbln3 is only found in the cerebellum and is not produced without the presence of either Cbln1 or 2 (Seigneur and Südhof, 2017). When Cbln1 was knocked out in the cerebellum, these mice displayed cerebellar synapse loss due to loss of presynaptic terminals. The cerebellum is an important region for motor functioning, and Cbln1 knockout caused impairment in motor skill acquisition and ataxia. (Rong et al., 2012; Hirai et al., 2005). Cbln1 was proven to be necessary for hippocampal learning through undetermined mechanisms (Otsuka et al., 2016). There have been very few studies investigating the functions of Cbln1 in the brain. However, Cbln2 and 4 have received much less attention. This project primarily focuses on elucidating the role of Cbln4.

1.4.1 Cerebellin4

Cbln4 is differentially produced in many brain regions, primarily the hippocampus, olfactory bulb, thalamus, prefrontal cortex, and the brainstem, among others. It also exhibits variable expression within many brain regions.

Cbln4 knockout mice were studied with regard to axon guidance and receptor interactions (Haddick et al., 2014; Wei et al., 2012) and not their function in

psychiatric disorders. Cbln1, 2 and 4 knock out caused seizures and abnormal motor behaviours (Seigneur and Südhof, 2018). Another study implicated Cbln4 in anxiety behaviours through the manipulation of the habenula to interpeduncular nucleus loop.

In the CA1 (cornu Ammonis 1) region of the hippocampus, Cbln4 was reported to localise at GABAergic synapses. They also found that GABAergic connection were reduced when Cbln4 gene was deleted through a shRNA-mediated deletion (Chacón et al., 2015). Cbln4 is expressed in specific GABAergic neurons of the hippocampus, and also highly expressed in the Entorhinal Cortex (EC). EC projections to the hippocampus form the first synapses in the dentate gyrus (DG) (Seigneur and Südhof, 2017).

In a recent report, Liakath-Ali et al., discovered that Cbln4 in the EC is required for EC → DG long-term potentiation (LTP) (Liakath-Ali et al., 2022).

Given these findings, it is likely that Cbln4 mediates hippocampal-dependent memory through its function in GABAergic synapse formation, and maintenance of hippocampal synaptic plasticity.

1.4.2 Cerebellins and psychiatric disorders

Jones et al. (2013) suggested that Cbln1 might contribute to increased susceptibility to Autism Spectrum Disorders (ASDs) during development. Clarke et al. (2012) demonstrated that mutations in Cbln2 were linked to both Tourette Syndrome (TS) and ASDs. Additionally, Lecourtier et al. (2004) identified mutations in Cbln4 in individuals with schizophrenia. Therefore, normal cognitive functioning relies on the proper operation of cerebellins, although the specific mechanisms of cerebellin function and their role in cognitive dysfunction remain unclear.

1.5 Learning and Memory and Adult Hippocampal Neurogenesis

1.5.1 Learning and Memory

Learning is defined as the acquisition of skill, while memory is the storage and expression of learned behaviour. Learning and memory can be quantified in animals using associative learning processes. Contextual fear conditioning is one such assay which utilises the associative learning capabilities of animals to train them to link an aversive (electrical shock) unconditioned stimulus (US) to the context (conditioned stimulus- CS) in which it is placed. Fear responses, such as freezing, reduction in

grooming and escape tendencies in mice, are thus studied. Learning and memory are crucial cognitive skills that are impaired in individuals with psychiatric disorders. Fear extinction is a phenomenon that occurs when the animal is repeatedly exposed to the context (CS) without the US, which diminishes the association between the CS and the US (measured by freezing responses here).

While synaptic plasticity in brain structures involved in learning and memory has been accepted as the cellular correlate of learning and memory, another mechanism of cellular plasticity that relies on adult neurogenesis could potentially mediate learning and memory (Snyder at al., 2005).

1.5.2 Adult Neurogenesis and Learning (Pattern Separation Memory)

Neurogenesis refers to "the creation of new neurons in the brain." The olfactory bulb and the subgranular zone (SGZ) of the DG are known to be sites of adult neurogenesis. Sahay et al. (2011b) proposed a function for these new neurons born in the DG. They help in the facilitation of learning and memory: "via distinguishing between overlaps in memory representations such that encoded information can be represented distinctly". This phenomenon is called pattern separation and is vital for spatial navigation.

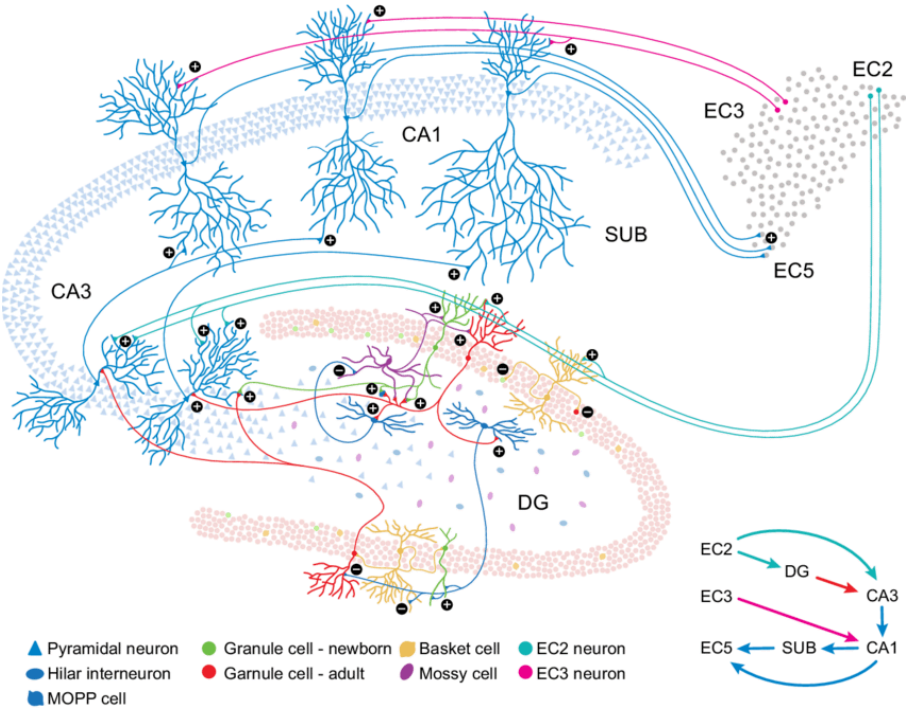

Fig. 1: The anatomy of the hippocampal circuit with new neuron integration. Granule cells in the DG connect to the CA3 region, which projects to the CA1 region. The CA1 in turn projects back to the EC and the subiculum. (image adapted from Aimone et al., 2014).

Adult hippocampal neurogenesis (AHN) has been linked to improving memory processes (Saxe et al., 2006; Leuner et al., 2002; Clelland et al., 2009). AHN can be regulated in mice in many ways - primarily, physical exercise and environment enrichment are shown to increase it; while environment aggravators are negative regulators. Mice housed in enriched environments displayed improved short term memory (Iso et al., 2007) and when subjected to voluntary physical exercise, they learnt faster in the Morris Water Maze (MWM) assay (van Praag et al., 1999). Rats exposed to prenatal stress showed a reduction in neurogenesis with an impairment in memory in the MWM assay (Lemaire et al., 2000).

Recent studies suggests that neurogenesis is associated with the forgetting of old memories. These studies propose that newly generated neurons play a role in shaping the hippocampal network to encode and retain memories, possibly necessitating the removal of past memories to enhance the brain's capacity for additional learning and memory formation.

In a groundbreaking study conducted by Akers et al. (2014), the relationship between memory retention and AHN was investigated. By comparing memory maintenance in elderly mice (with low neurogenesis) to that in younger mice (with high neurogenesis) following fear conditioning and subsequent fear recall tests over varying time intervals from 1 day to 6 weeks, they made significant observations. While older mice were able to display unimpaired recall of the fear memory, the younger mice exhibited a rapid reduction after just seven days. Previous studies have shown that wheel running in mice or administration of fluoxetine, an antidepressant, increases AHN. Utilising this, they found that adult mice displayed an improved memory. Conversely, decreasing AHN in younger mice actually enhanced their memory conservation. Guinea pigs and degus (small rodents) have previously been shown to have diminished rates of neurogenesis. The study thus utilised the special features of these animals to find out if neurogenesis and forgetting were correlated. They reported that these animals displayed proper memory functioning, however, this was reversed when neurogenesis was increased through the administration of memantine. These findings, along with other studies, suggest that AHN is associated with forgetting and plays a critical role in learning and memory processes, particularly in spatial navigation tasks.

Nakashiba and colleagues (2012) showed that while mature neurons are important for context separation of distinct conditions, younger neurons are essential for finer discrimination of extremely similar nature. Studies have shown that inhibiting AHN impaired spatial pattern separation (Tronel et al., 2012; Scobie et al., 2009; Clelland et al., 2009). Previous findings report that EC→DG circuitry is also involved in pattern separation (McHugh et al., 2007; Gilbert et al., 2001). Another study found that increased adult neurogenesis was sufficient to improve pattern separation abilities

(Sahay et al., 2011a), and also reduced depression-like and anxiety behaviours (Hill et al., 2015).

Although AHN is undoubtedly involved in pattern separation (Niibori et al., 2012; Creer et al., 2010), the precise course of functioning is unknown. A study done in humans found that physical exercise improved pattern separation abilities compared to those who did not exercise (Déry et al., 2013). Aging is linked to reduced AHN (Knoth et al., 2010), and a study found that there is reduced pattern separation ability due to the effects of aging (Stark et al., 2013).

Many brain regions input to the DG, like the EC, subiculum, locus coeruleus, septal nuclei and the supramammilary area of the hypothalamus (Amaral et al., 2007). There is an impairment of LTP in the Cbln4 EC → DG circuit (Liakath-Ali et al., 2022) and previous work from the lab showed that Cbln-4 deletion from the EC→DG circuit impaired the pattern separation skill in mice (unpublished data).

Ya-Dong Li et al. (2022) showed that inputs from the supramammillary nucleus (SuM) to the DG are vital for the production and maturation of newborn neurons. Through glutamate transmission, SuM-DG projections promote neural stem cell activation. They also showed that direct SuM GABA transmission onto the DG interneurons is necessary for the neural stem cell differentiation and regulation of dendritic growth of newborn neurons.

All this evidence thus points to the role that Cbln4 could play in cognitive skills such as learning, memory and pattern separation. Cbln4 is likely to play a crucial role in AHN and regulating the basal synaptic properties of DG granule cells.

Hypotheses and Aims

The DG is crucial for pattern separation, while Cbln4 is important for LTP in the EC→DG circuit. Inputs from the SuM and EC are found to be vital for the promotion and modulation of AHN. The DG interneurons also regulate the development and activity of immature neurons. In the present study, we want to explore the function of Cbln-4 in pattern separation and adult hippocampal neurogenesis via Cbln4 deletion from presynaptic inputs to the DG. We also want to pick out the function of Cbln-4 in the DG and its consequence on fear learning and extinction.

Aims:
1. To investigate the role of Cbln4 in mediating pattern separation memory

2. To examine the role of Cbln4 in facilitating steady state and experience-dependent neurogenesis in the DG
3. Examine the role of Cbln4 in determining the synaptic properties in the hippocampus.

Chapter 2 Materials and Methods

2.1 Mice:

All rodent experiments were performed in accordance with protocols approved by the NUS Institutional Animal Care and Use Committee (IACUC). The design of the targeting for the generation of Cbln4 cKO mice was as previously described (Seigneur & Sudhof, 2017). Mice were bred using standard procedures and were maintained on a hybrid C57/Bl6 background. The mice were maintained on a 12:12 light-dark cycle with lights on at 0700.

2.2 Stereotactic Surgery:

Postnatal 21 to 30-day-old Cbln4 cKO mice were anaesthetised with ketamine/medetomidine cocktail (75 mg/kg body weight ketamine and 1mg/ kg body weight medetomdine) by intraperitoneal injection (i.p.) and subsequently buprenorphine (analgesic) (0.1 mg/kg body weight, subcutaneous (s.c.). Animals' heads were shaved once they were in a state of deep anasthesia. The surgical site was cleaned with iodine and 70% ethanol in alternating rounds over three times. Ophthalmic ointment was also applied to avoid injury and drying of the eyes. Mice were immobilised on a stereotactic apparatus and stability was adjusted to ensure delivery of virus would take place in the precise region of interest. Mice were injected with a retrograde Adeno Associated Virus (AAVs) driving either rAAV-Cre (hSyn-rAAV-Cre-GFP) or the inactive rAAV-ΔCre (hSyn-rAAV-ΔCre-GFP) at coordinates: -1.6 mm Anterior-Posterior (AP), ± 1.4 mm Medial-Lateral (ML), and -1.8 to 2.00 mm Dorsal-Ventral (DV); and -2.00 mm AP, ± 1.5 mm ML to bregma at a depth of -1.8 mm to -2.00 mm. AAVs were injected using a glass cannula and monitored by a Harvard Apparatus injection pump at a flow rate of 150 nL/min. A total volume of 500 nL was injected in each of the four above mentioned sites to cover the entirety of the dorsal DG. The glass cannula was removed slowly and the skin was sealed with simple uninterrupted sutures. Atipamezole (1 mg/kg body weight, i.p.) was injected to reverse the effects of the anasthesia. Animals were placed in clean cages with moist food pellets on the bottom of the cage for easy access. Buprenorphine (analgesic) (0.1 mg/kg body weight, s.c.) was injected for three days post-surgery to reduce any pain felt by the animals and were closely monitored.

2.3 EdU Injections:

Animals were rested for 17 days after the surgery. Each day, thirty minutes before the beginning of the contextual fear discrimination task (Day 0 to 14), mice were given an i.p. injection of 5-Ethynyl-2′-deoxyuridine (EdU) (50 mg/kg body weight) to label the dividing cells.

2.4 Contextual Fear Discrimination (Pattern Separation) Task:

Behavioural experiments were conducted on mice seventeen days post-surgery. This behavioural paradigm evaluates the animals' ability to differentiate between two closely resembling contexts (but not identical) found to engage the DG. The two similar contexts (training context – Context A and Similar context – Context B) share many features, including an exposed stainless steel grid floor and roof. Context B differed from Context A with respect to chamber lights (OFF) and fan (ON), two slightly slanted acrylic inserts on opposite sides of the grid, and the door left ajar. 70% ethanol was used to clean the grids and plastic inserts between trials in Context A, while clidox was used for Context B - this is to provide slight odour discrimination between the two contexts. Mice were accustomed to the behavioural room for thirty minutes before the assay began. On day 0, following a 3-minute habituation in context A, mice receive three-foot shocks in this chamber. This was followed by 14 days of fear discrimination learning in which they were exposed to each context for 180s but received a single foot shock at the end of the session only in Context A, but not in Chamber B. Mice were left for an extra 15s in the training chamber (Context A) to associate the shock to the context. After resting for 1 hour in a separate room, mice were tested in Context B and received no shock. Freezing levels were measured for 180s in both contexts to assess their discrimination indices. (adapted from Sahay et al., 2011a).

2.5 Contextual Fear Conditioning and Extinction:

Behavioural experiments were conducted on mice twenty one days post-surgery. Mice were habituated to the behavioural room for 30 minutes before the testing began. On day 0, following a 3-minute habituation in context A, mice receive three foot shocks in this chamber. This was followed by four days of extinction learning, where the animals were exposed to the training context but did not receive any footshock. On

days 1 and 4, animals were also placed in a novel context, which was distinct from the original chamber.

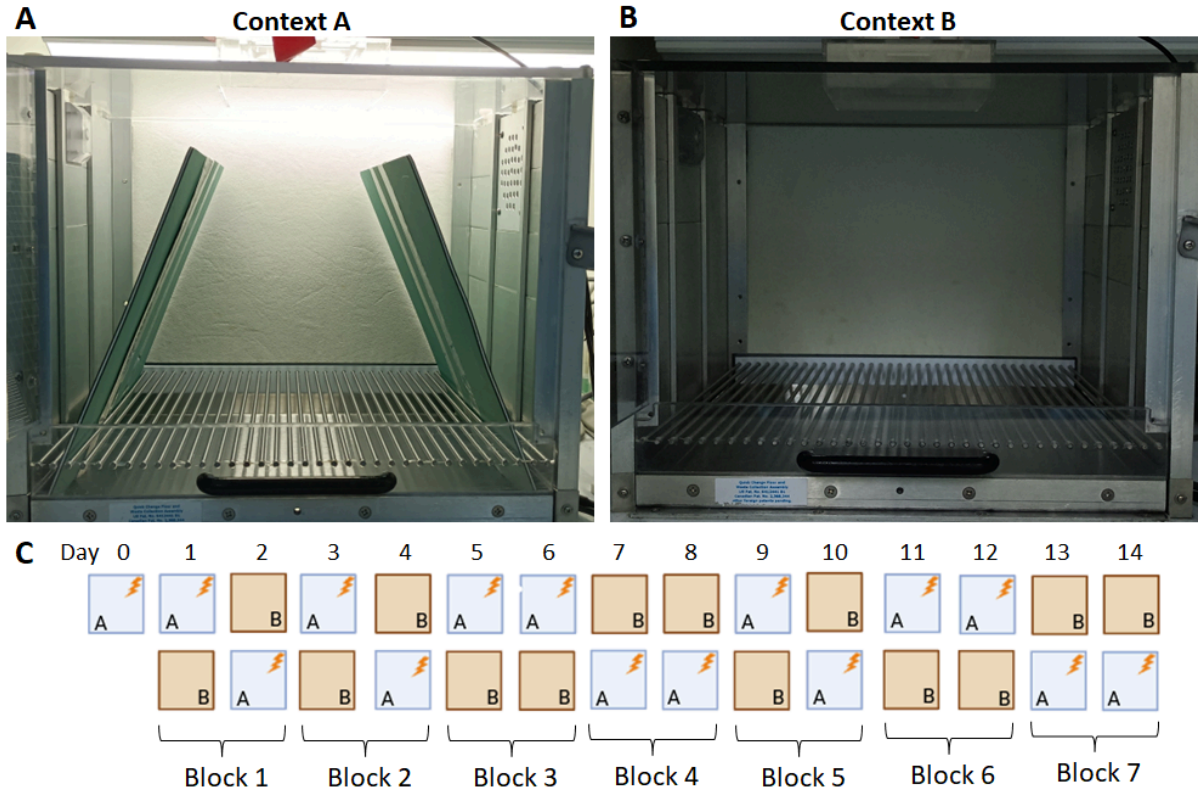

Fig. 2: Contextual fear discrimination assay protocol: Representative images of the experimental set-up for the contextual fear discrimination assay: (A) Context 'A': Lights on, tilted plastic inserts on opposite ends of the fear chamber, chamber cleaned with 70% ethanol and doors were shut completely; (B) Context 'B': Lights off, chamber cleaned with Clidox and doors were left ajar; (C) Timeline of the assay: freezing patterns are averaged over two day blocks.

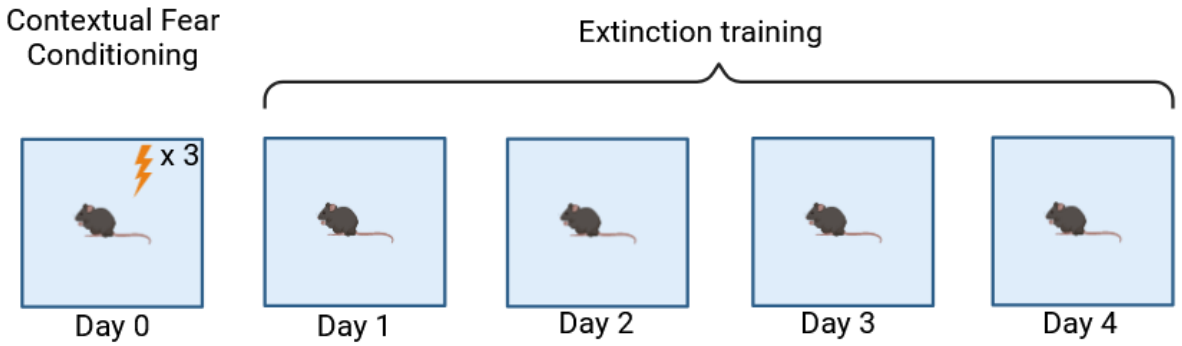

Fig. 3: Timeline of the Contextual Fear Conditioning and Extinction Paradigm.

2.6 Mouse brain isolation and sectioning:

Mice were humanely euthanized with halothane, and the bodies were pinned to a board using thumbtacks. Careful incisions were made to reveal the diaphragm, and

eventually the heart to locate the left ventricle. By inserting the needle into the left ventricle of the heart, animals were perfused with chilled 10 mL 1X Phosphate buffered saline (PBS) followed by 10 mL chilled 4% Paraformaldehyde (PFA). Animals were beheaded to remove the brains carefully and were placed in 4% PFA at 4°C overnight. The next day, the brains were placed in 1X PBS for further processing. Coronal brain sections, 50 µm thick, were obtained using a vibratome. Sections containing areas of interest related to neurogenesis, such as the dorsal hippocampus, as well as input regions to the dentate gyrus like the hypothalamus and entorhinal cortex, were collected in 24-well plates containing 0.03% Sodium Azide in PBS and kept aside for subsequent immunostaining.

2.7 Immunostaining:

ClickTech In Vivo EdU Cell Proliferation Kits for Imaging: Dye 594 (Merck, USA) was used to stain for EdU following the manufacturer's instructions: two washes with 3% BSA | PBS for 20 minutes, one wash with 0.5% PBST for 20 minutes, two washes with 3% BSA | PBS for 20 minutes, followed by 500µl click reaction mixture (Deionised water: 379µl; Reaction Buffer: 50µl; Reactor System: 20µl; Dye Azide: 1µl; Buffer additive: 50µl) per well. Each well was then washed three times with 3% BSA | PBS for 20 minutes.

Sections were then stained for doublecortin (Dcx). Wells were washed with 10% Goat Serum | 0.2% PBST for one hour and then incubated overnight with doublecortin primary antibody (ab18723; Abcam, UK) at 1:1000 dilution in 10% Goat Serum | 0.2% PBST. The next day, wells were washed three times with 0.2% PBST for twenty minutes, followed by secondary antibody 1:1000 Alexa-Fluor 647 goat anti-rabbit (A-21245, Invitrogen) and stained with Hoescht 33342 (62249, ThermoFisher) at 1:1000 dilutions in 10% Goat Serum | 0.2% PBST. Wells were then washed three times with 0.2% PBST for twenty minutes and three times with PBS for 5 minutes. The sections were then placed onto microscope slides (Continental Lab products) and covered with Aqua-PolyMount Coverslipping Medium.

2.8 Quantification for EdU and Dcx:

Three sections from the beginning, middle, and end regions of the dorsal hippocampus were chosen for each animal. Images were obtained on a confocal microscope (FV3000RS Zeiss) and analysed using ImageJ software.

Images were loaded onto ImageJ software, and each channel was split into a separate window. The region of interest (Dentate Gyrus) in the image was cropped using the Hoechst marker and duplicated for further processing. The 3D objects counter tool was used in the software analysis function. The size of cells to be quantified was calculated from the Hoechst staining during standardisation. Manual thresholding was done to decrease noise in the background, and an '*objects map'* and '*statistics'* were obtained for each processed image with EdU and Dcx markers. The number of positive cells was averaged as cells per 50µm sections and then extrapolated to the thickness of the dorsal dentate gyrus (1mm) (Amaral et al., 2007).

2.9 Electrophysiology:

Twenty one days post surgery, animals were deeply anaesthetised with isoflourane. Careful incisions were made to reveal the diaphragm and eventually the heart to locate the left ventricle. The needle was inserted into the left ventricle of the heart and 10ml cutting solution ((in mM) 228 sucrose, 26 NaHCO3, 11 glucose, 2.5 KCl, 1 NaH2PO4, 7 MgSO4 and 0.5 CaCl2) was used for the transcardial perfusion. Animals were beheaded and the brains were carefully and quickly taken out and placed in ice-cold, cutting solution with a high sucrose concentration. Leica vibratome was used to cut 280 µm sections in the cutting solution containing high sucrose concentration. The sections were then quickly placed in an incubation chamber with artificial cerebrospinal fluid (ACSF) containing (in mM) 119 NaCl, 26 NaHCO3, 11 glucose, 2.5 KCl, 1 NaH2PO4, 1.3 MgSO4 and 2.5 CaCl2. The incubation allowed the sections to recover at 34°C for thirty minutes. After the incubation, the beaker containing the sections was removed from the incubation chamber and placed in room temperature for another hour. To record from the sections, the recording chamber was constantly flowing with heated ACSF (28–30°C) and continuously gassed with 95% O2 and 5% CO2. For voltage-clamp recordings, borosilicate glass pipettes (3–5 MΩ) were filled with a Cesium internal solution containing (in mM) 135 CsMeSO4, 8 NaCl, 10 HEPES, 0.25 EGTA, 2 Mg2ATP, 0.3 Na3GTP, 0.1 spermine and 7 phosphocreatine (pH 7.25–7.3; osmolarity 294–298). Data was acquired with a MultiClamp 700B amplifier (Axon Instruments) and digitised at 8 kHz using an ITC-18 A/D converter (Instrutech Corporation) and filtered at 4 KHz. Data were acquired and analysed using Axograph-X (Axograph).For mini IPSC recodings, ACSF was supplemented with 20µM NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione - AMPA receptor antagonist) 25µM AP5 (2-amino-5-phosphonopentanoic acid - NMDA receptor antagonist) and 0.1µM TTX (Tetrodotoxin - Sodium channel blocker). Cells were recorded in voltage clamp at a holding potential of 0mV. Recordings were filtered at 2000Hz, and the synaptic current decays were fitted with a double exponential equation of the form: $I(t) = \exp(-t/\tau_f) + \exp(-t/\tau_S)$, and τf and τs are their respective decay time constants. The template baseline was 5ms, and the

length was 30ms. Events were fitted to two templates, with amplitude 8pa and rise time constant 0.1ms common for both templates, while the decay time constant was 10ms and 20ms - to account for fast and slow decay rates. Individual episodes were manually reviewed, and events that were falsely detected were not considered for further analysis.

Chapter 3 Results

3.1 Verifying the efficacy of the pattern separation assay

An experiment for the contextual fear discrimination task was performed using four control Cbln4-cKO animals to ascertain the potency of the pattern separation task. The animals were naive and had not undergone surgery previously.

On day 0, mice were placed in the fear conditioning chamber, specifically, Context A and were subjected to fear conditioning (FC) training. To condition the mice to associate the footshock to context A, the mice were habituated to the fear chamber for 180 seconds, followed by three foot shocks (two seconds each) delivered every 2 minutes. Fear conditioning memory was measured as the percentage of time the mice displayed freezing behaviour when foot shock was not administered (% freezing).

On subsequent days, mice were exposed to Context A (Shock) and Context B (No Shock) each day in a pseudo-random manner (Fig. 2). The assay was designed to train the mice to differentiate between the two similar (but not identical) contexts. Success in the pattern separation task would be assessed by observing a progressive rise in freezing behavior in context A (shock context) and a decline in freezing behavior in context B (no shock context) over a period of 14 days, referred to as the discrimination index. The discrimination index here is calculated as % freezing in context A divided by the sum of % freezing in context A and % freezing in context B ([A/A+B]). A discrimination index significantly exceeding 0.5 would indicate a preference for freezing in context A, implying proficient pattern separation abilities. The discrimination index here is averaged over 2-day blocks.

The two contexts are quite similar, and the assay intends to train the mice to discriminate between them. They are expected to show a higher freezing behaviour in Context A (Shock) and converse in Context B (No shock). Initially, the mice were at a discrimination index of 0.6 (0.6 $\pm$ 0.02), and as the training progressed, they gradually peaked at 0.72 (0.72 $\pm$ 0.07) on the last block (Block 6: Days 11 and 12) (Fig. 4). The percentage freezing in contexts A and B is also plotted (Fig. 4C), showing an overall decrease in freezing behaviour in both contexts as the training progresses. However, notably, the reduction in freezing in context A is also accompanied by a higher decrease in freezing in context B, thus improving their discrimination index. This experiment shows that the contextual fear discrimination assay is potent in measuring the ability of animals to test the pattern separation ability in mice.

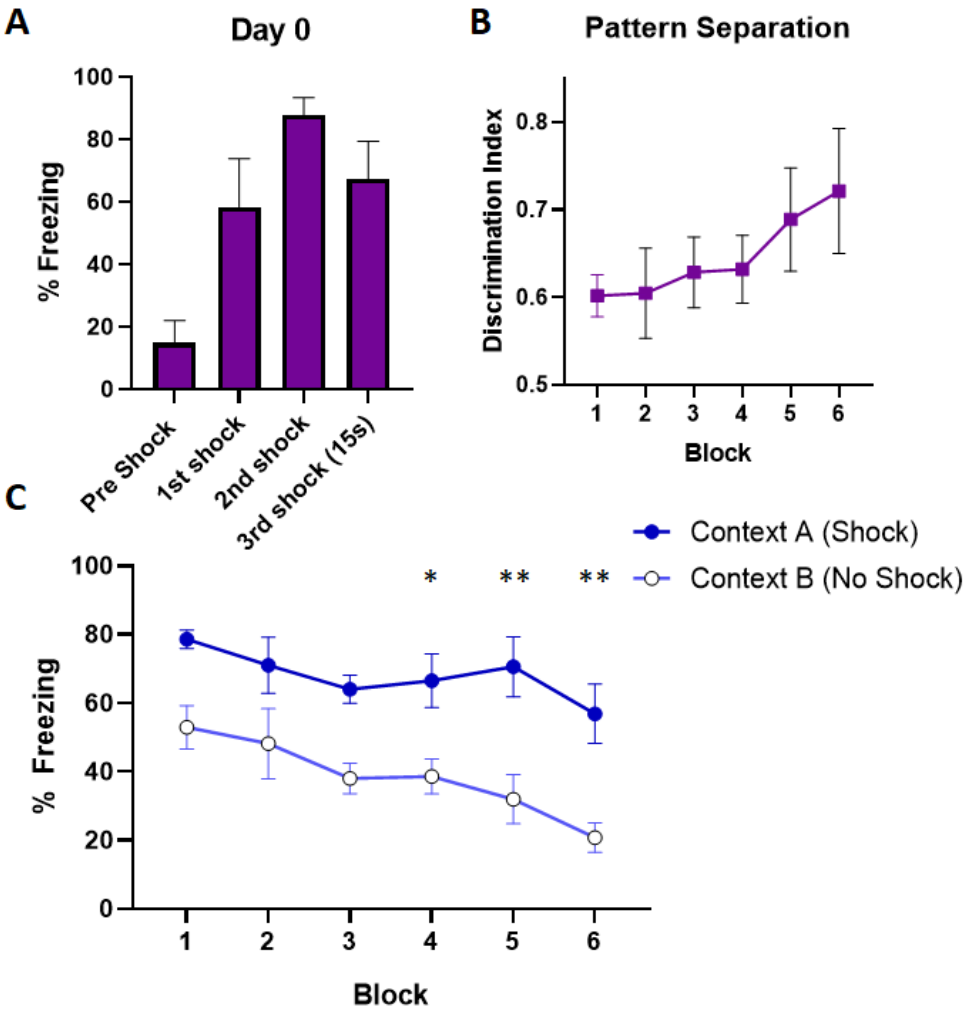

Fig. 4: Verifying the feasibility of the contextual fear discrimination task: (A) Contextual fear conditioning on Day 0 in context 'A' (B) Two-day average blocks of discrimination index (% freezing in 'A' divided by the sum of % freezing in 'A' and 'B'. (Block 1 = 0.60 ± 0.02, Block 6 = 0.72 ± 0.07); (C) Two-day average blocks of freezing patterns of animals in contexts 'A' and 'B' during pattern separation test. Freezing behaviour in contexts A and B during pattern separation task: Block 1: Context A: 78.61 ± 2.64%; Context B: 52.95 ± 6.321%; Block 6: Context A: 56.89 ± 8.63%; Context B: 20.77 ± 4.34%. All data are represented as mean ± standard error of the mean (SEM). 2-way ANOVA repeated measures was used to analyse the data. Sidak's post-hoc tests were performed, where *p<0.05, **p<0.01; n =4.

3.2 Conditional knockout of Cbln4 from the DG

Subsequently, our objective was to examine the impact of presynaptic inputs from Cbln4 neurons to the DG on the pattern separation ability in mice. To achieve this, we utilised a retrograde-AAV to selectively eliminate Cbln4 from presynaptic inputs to the DG. The regulation of Cbln4 expression in these inputs was achieved by stereotaxic injection of AAVs carrying either rAAV-Cre or rAAV-ΔCre (serving as a

non-functional truncated control) into the dentate gyrus (DG) of Cbln4-mVenus-tdTomato conditional knockout (cKO) mice (see Fig. 5).

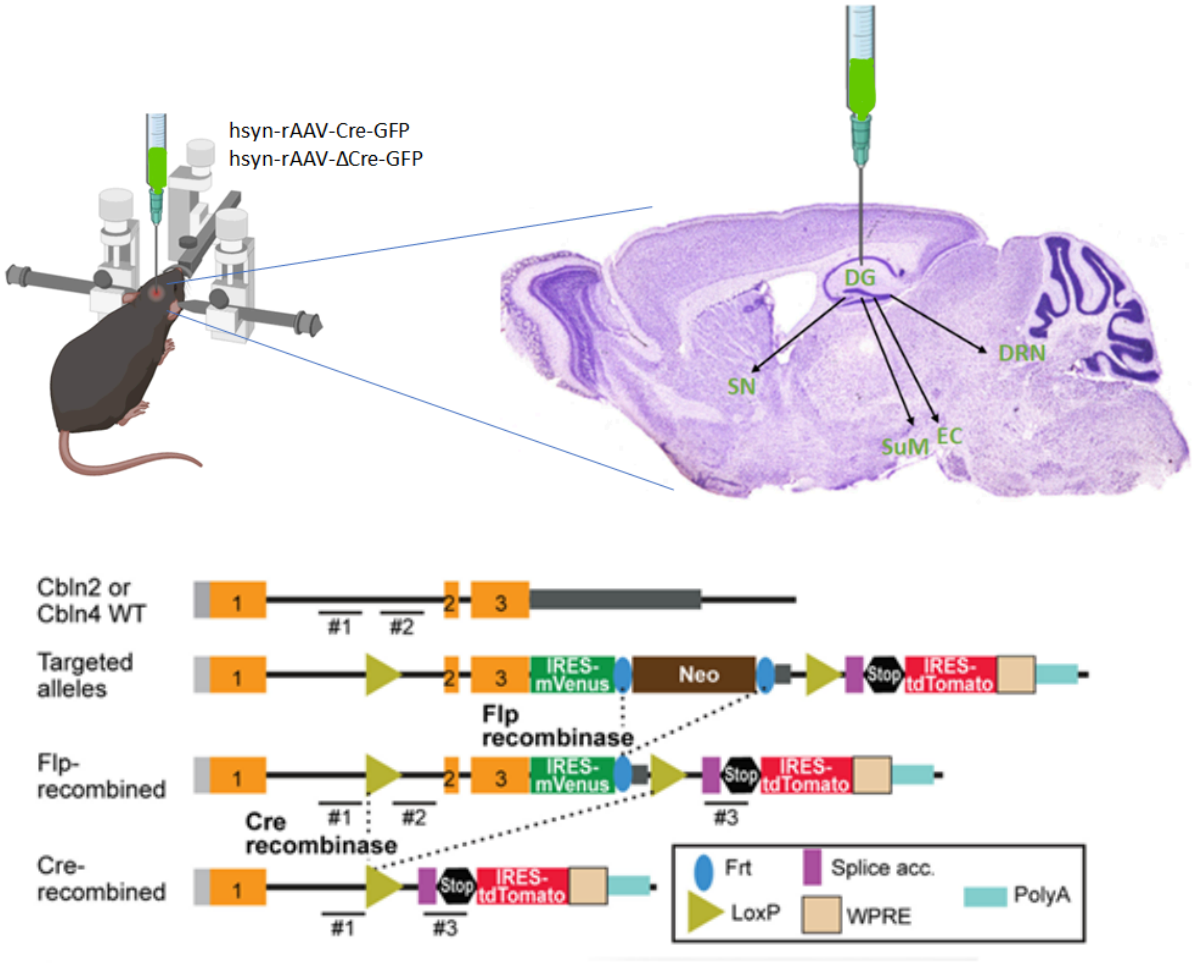

Fig. 5: Conditional deletion of Cbln4 from presynaptic inputs to the DG using AAVs. (A) Diagram illustrating the delivery of hSyn-rAAV-Cre-GFP (experimental) or hSyn-rAAV-ΔCre-GFP (control) into the dentate gyrus (DG) of Cbln4 cKO mice via stereotactic injection. Brain regions with Cbln4 expression and retrogradely labelled with the AAVs are highlighted in green. (B) An illustration of the Cbln4 cKO model with exons and introns is highlighted. Deletion of exons 2 and 3 following cre-recombinase delivered via stereotaxic surgery (Siegneur & Sudhof, 2017).

To verify the modification of presynaptic Cbln4 expression in the dentate gyrus (DG) following the stereotaxic injection of retro-AAVs carrying hSyn-rAAV-Cre-GFP and hSyn-rAAV-ΔCre-GFP, verification of the injection sites, targeting both the hemispheres of the DG, was conducted (Fig. 6). GFP was utilized as a marker in both groups of animals. Furthermore, presynaptic inputs to the DG, which include Cbln4, were examined to confirm the presence of GFP in regions such as the supramammillary nucleus (SuM) and entorhinal cortex (EC).

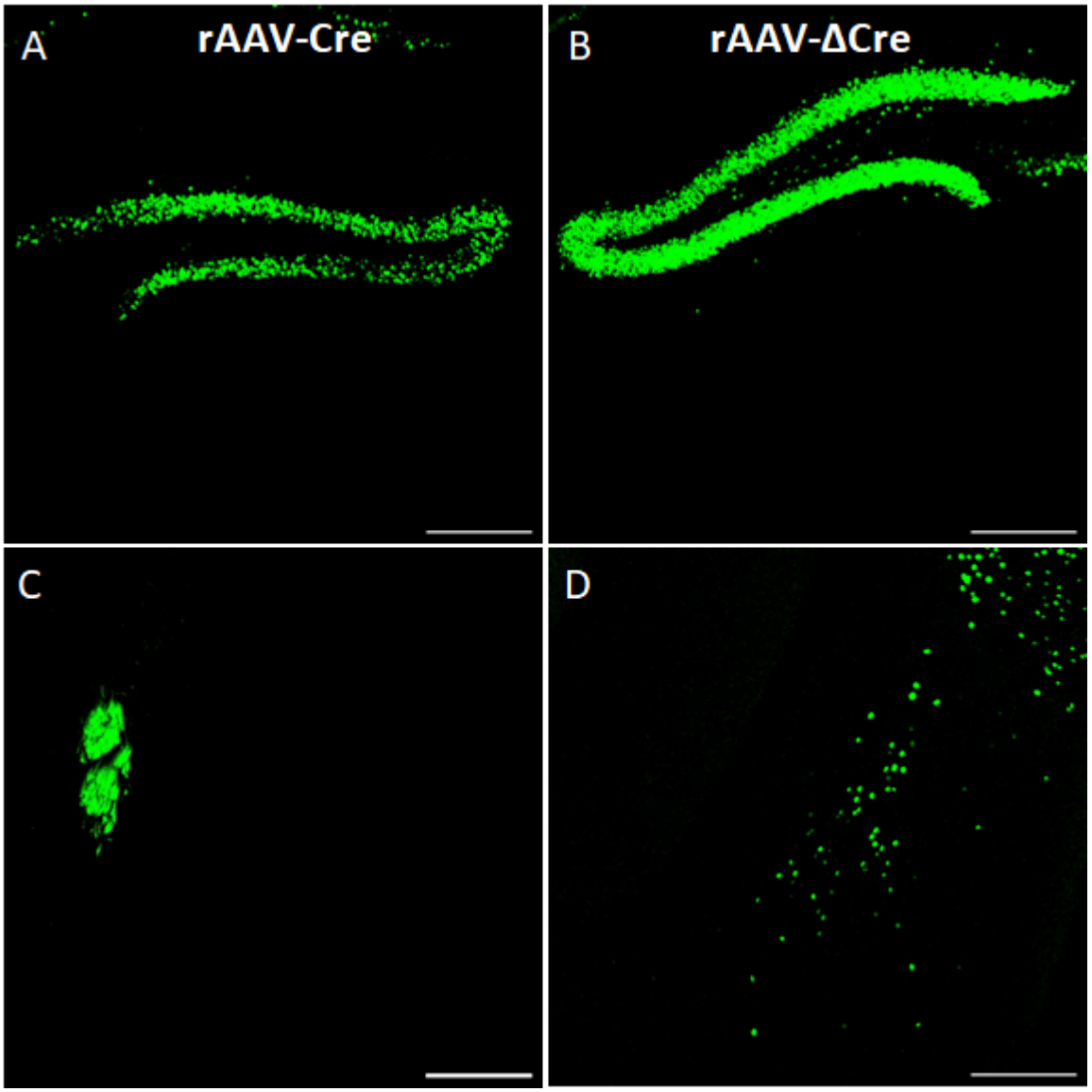

Fig. 6: Verification of AAV injection accuracy into the DG. Illustrative images of (A) rAAV-Cre expression in the DG, or (B) rAAV-ΔCre expression in the DG, (C) Retrogradely infected SuM neurons expressing Cre-GFP (D) retrogradely infected Cre-GFP expression in the EC. Scale bar: 250μm

3.3 Cbln4 from presynaptic inputs to the DG is not necessary for hippocampal fear conditioning

Cbln4 cko mice injected with rAAV-Cre (Cbln4 deletion) or rAAV-ΔCre (control) were subjected to the contextual fear discrimination task as described above. Both groups of mice were subjected to fear conditioning (FC) training in the fear conditioning chamber with context A on Day 0. No significant difference was observed between rAAV-Cre and rAAV-ΔCre mice groups in their % freezing over the three shocks delivered on Day 0. Their baseline freezing (180 seconds habituation)

was about 6%, with a gradual increase in freezing. After the third shock, both groups of animals displayed approximately 80% freezing (Fig. 7). The consistent freezing behaviour patterns observed in both sets of animals indicate that the acquisition of FC memory was effectively achieved and showed no significant alteration as a result of Cbln4 deletion.

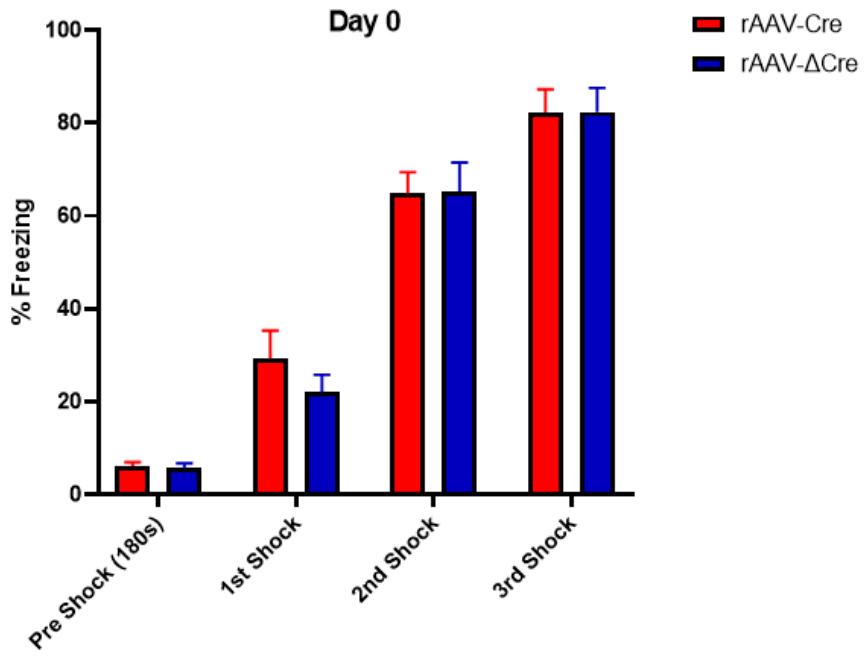

Fig. 7: Cbln4 from presynaptic inputs to the DG is not necessary for hippocampal fear conditioning (FC) (A) Retrograde deletion of Cbln4 from the DG (rAAV-Cre; n = 15) and control (rAAV-ΔCre; n = 16) does not affect acquisition of contextual fear memory as assessed by % freezing on Day 0. Pre Shock (180s): rAAV-Cre = 6.2 ± 0.81%, rAAV-ΔCre = 5.77 ± 1.01%; 1st Shock: rAAV-Cre = 29.3 ± 6.04%, rAAV-ΔCre = 22.05 ± 3.79%; 2nd Shock: rAAV-Cre = 64.84 ± 4.58%, rAAV-ΔCre = 65.30 ± 6.20%; 3rd Shock: rAAV-Cre = 82.28 ± 4.98%, rAAV-ΔCre = 82.39 ± 5.16%. All data are represented as mean ± standard error of the mean (SEM). (rAAV-Cre: n =15, rAAV-ΔCre: n =16).

3.4 Deletion of Cbln4 from inputs to the DG leads to deficits in pattern separation memory

From Days 1 to 14, the mice went through a contextual fear discrimination assay (pattern separation test). They were exposed to both Contexts A and B daily in a pseudo-random manner as described above. Notably, the shock was delivered solely in Context A, with Context B serving as a non-shock environment.

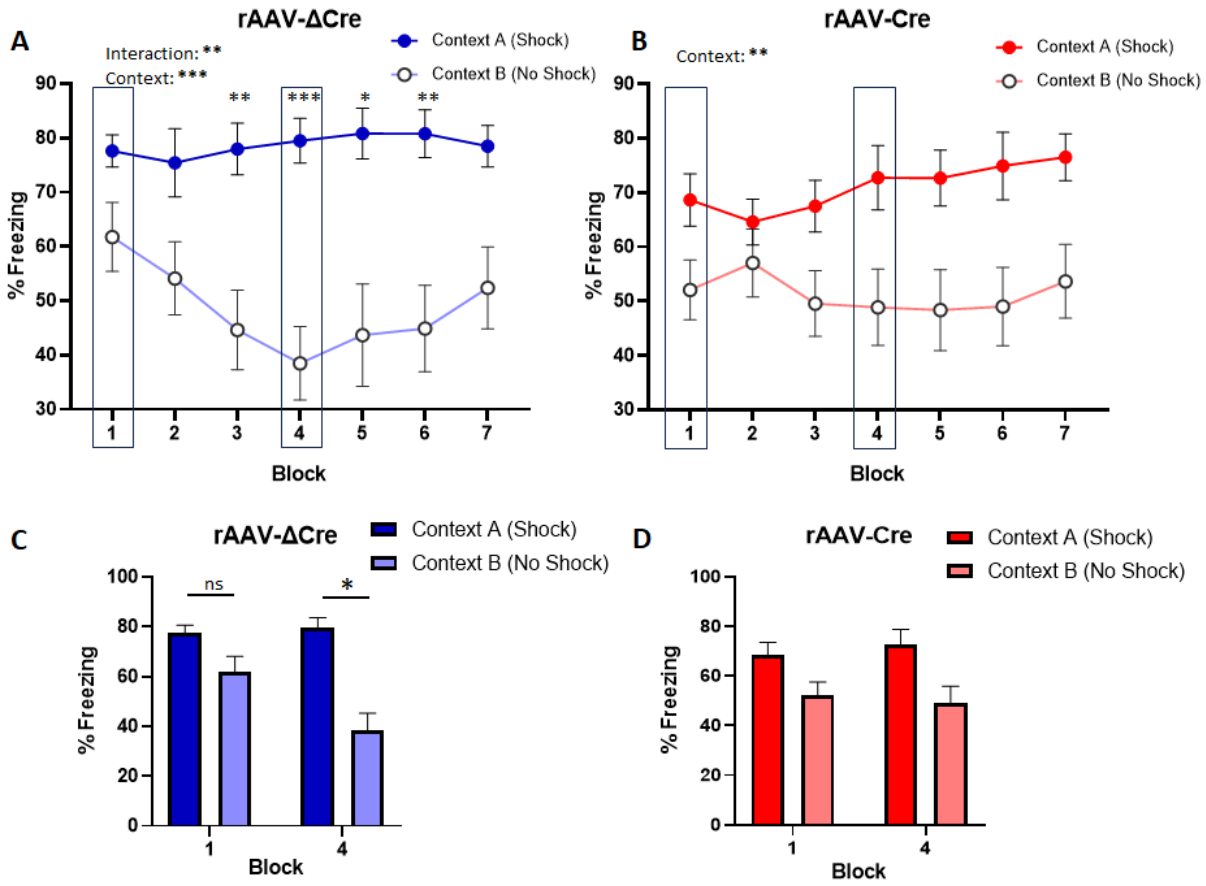

Fig. 8: Retrograde deletion of Cbln4 from the DG results in an inability to differentiate between two similar contexts during the retrieval of memories (A,B) Two-day average blocks of freezing patterns of both groups of animals in contexts A and B during pattern separation test. Freezing behaviour in contexts A and B during pattern separation test on (C) Block 1, rAAV-ΔCre: Context A = 77.60 ± 2.99%; Context B = 61.75 ± 6.37%; rAAV-Cre: Context A: 67.16 ± 4.61%, Context B: 52.05 ± 5.56% (D) and on Block 4, rAAV-ΔCre: Context A = 79.48 ± 4.12%; Context B = 38.47 ± 6.78%; rAAV-Cre: Context A: 67.77 ± 7.32%, Context B: 52.30 ± 6.17%. All data are represented as mean ± SEM. 2-way ANOVA repeated measures was used to analyse the data. Sidak's test was used to do the post-hoc tests only when the interaction was significant, where *p<0.05, **p<0.01, ***p<0.001 (rAAV-Cre: n =10, rAAV-ΔCre: n =11).

Freezing behaviour in Contexts A and B is depicted as percentages and averaged over 2 day blocks (Fig. 8). The control group maintained a steady freezing behaviour in Context A while freezing behaviour declined gradually in Context B (Fig. 8A and 8B). There is a significant difference in freezing behaviour between the two contexts of the control group from blocks 3 to 7 (Block 3: p = 0.0095, Block 4: p = 0.0006, Block 5: p = 0.0221, Block 6: p = 0.0087, Block 7: p = 0.0529). This was not the case with the rAAV-Cre animals, as they displayed a slight increase in freezing in context A, while maintaining freezing in context B.

This can be visualised in another graph displaying the discrimination index Both groups of mice began at similar levels of discrimination index in the first block

(approximately 0.57). The rAAV-ΔCre animals displayed higher levels of discrimination until the fourth block compared to the rAAV-Cre group (Block 4: rAAV-ΔCre: 0.69 ± 0.03, rAAV-Cre: 0.60 ± 0.03). This was followed by a gradual decrease in the discrimination index for the rAAV-ΔCre down to the levels of the rAAV-Cre group (Block 7: rAAV-ΔCre: 0.60 ± 0.05, rAAV-Cre: 0.60 ± 0.03). The rAAV-Cre group displayed very little increase in discrimination index through all the blocks (rAAV-Cre: Block 1: 0.57 ± 0.02, Block 6: 0.61 ± 0.03) while the rAAV-ΔCre group displayed an increase in the discrimination index (rAAV-ΔCre: Block 1: 0.57 ± 0.03, Block 4: 0.69 ± 0.03) (Fig. 9). The lack of decrease in freezing behaviour in context B (Fig. 8B) most likely contributed to the lack of increase in the discrimination index for the rAAV-Cre group.

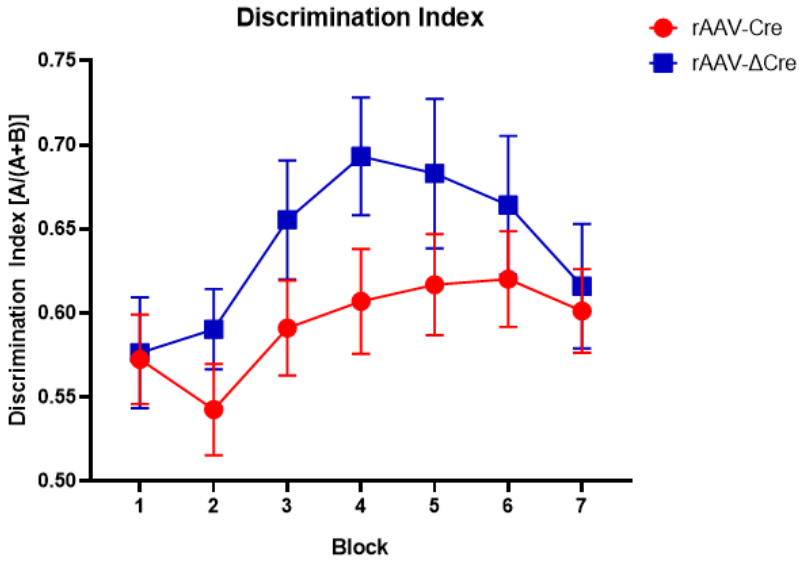

Fig. 9: Two-day average blocks of discrimination index [A/(A+B)]. Block 1: rAAV-Cre = 0.57 ± 0.02, rAAV-ΔCre = 0.57 ± 0.03; Block 4: rAAV-Cre = 0.60 ± 0.03, rAAV-ΔCre = 0.69 ± 0.03. All data are represented as mean ± SEM. (rAAV-Cre: n =10, rAAV-ΔCre: n =11).

This data shows that the control group displays a higher rate of increase in the discrimination index, indicating success in pattern separation capabilities, while the rAAV-Cre group did not possess this ability. Thus, Cbln4 deletion from presynaptic inputs to the DG leads to a lack of pattern separation abilities in mice, indicating the essential role of Cbln4 inputs to the DG.

3.5 Experience-dependant adult neurogenesis in the dorsal Dentate Gyrus

After the pattern separation task was completed, both animal groups were evaluated for their corresponding neurogenesis rates in the DG of the Dorsal hippocampal

region. 5-Ethynyl-2′-deoxyuridine (EdU) is a thymidine analogue that labels mitotic cells. EdU was administered each day, 30 minutes before the start of the first behavioural session each day. We also stained for doublecortin (Dcx), a protein that labels immature neurons and neural precursors (Fig 9A and 9B). The counts of EdU+ and Dcx+ cells served as readouts for the neurogenesis levels during the pattern separation assay.

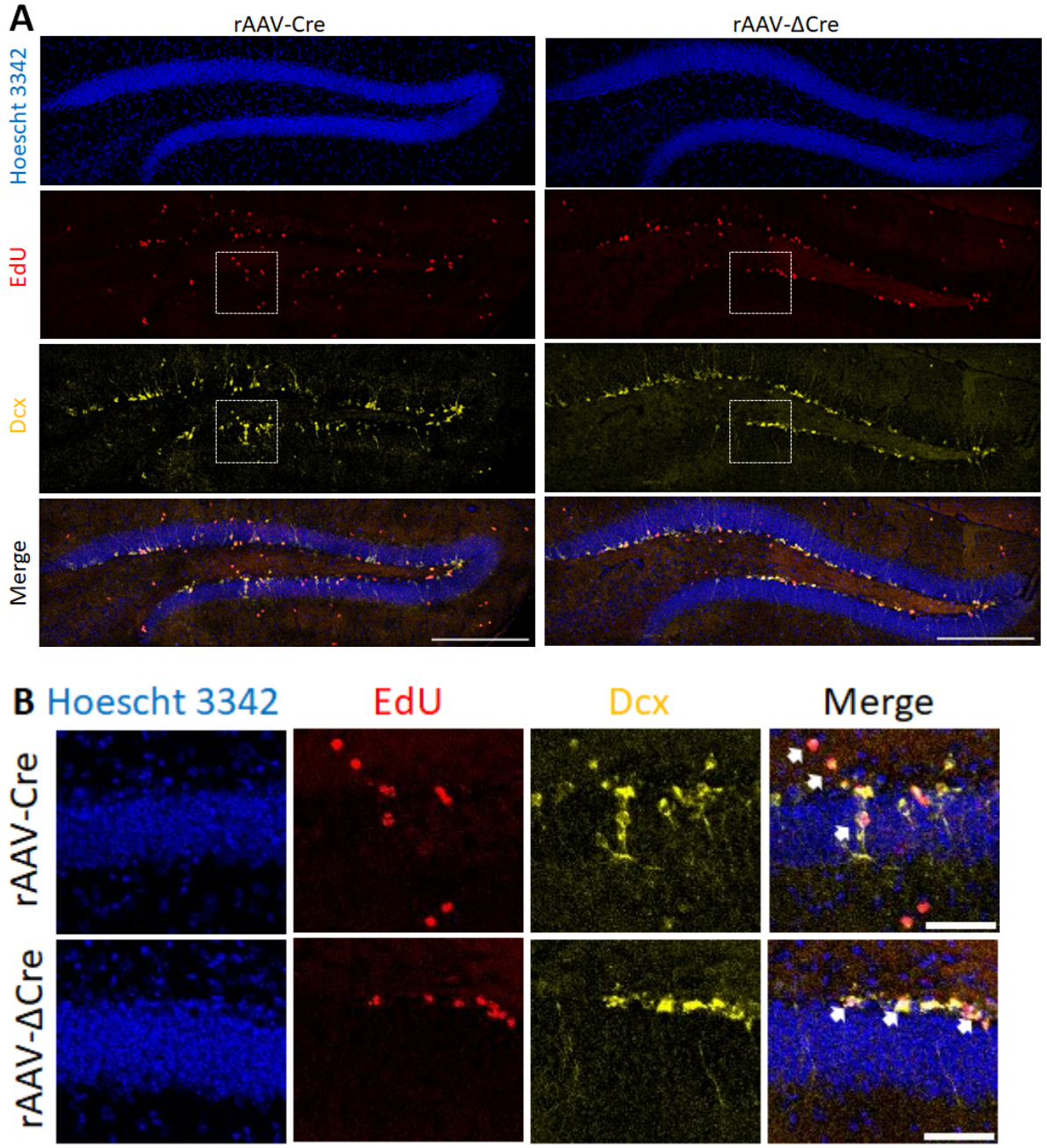

Fig. (10): Illustrative staining images of the dorsal DG. (Left) rAAV-Cre and (Right) rAAV-ΔCre (A) From top to bottom, Hoescht 33342 for soma staining (488 nm), EdU^{+} cells (594 nm), Dcx^{+} cells (647 nm) (pseudocolour yellow), composite photo. Scale bar = 250 µm. (B) Zoomed-in pictures of (Top) rAAV-Cre (Bottom) rAAV-ΔCre dorsal DG indicated by the rectangular boxes in Figure 9A. Scale bar = 50 µm.

The first batch of animals tested for neurogenesis had undergone both FC and PS. We found a significant increase in the number of both, EdU^+ and Dcx^+ cells per DG of each animal injected with rAAV-Cre compared to rAAV-ΔCre (Fig. 11B and C) (EdU: FC + PS: $p = 0.0127$; DcX: FC + PS: $p = 0.0137$). Previous studies have linked AHN to improve pattern separation abilities. Contrastingly, our study reports that the pattern separation deficit observed in the rAAV-Cre group of animals is coupled with an increase in AHN.

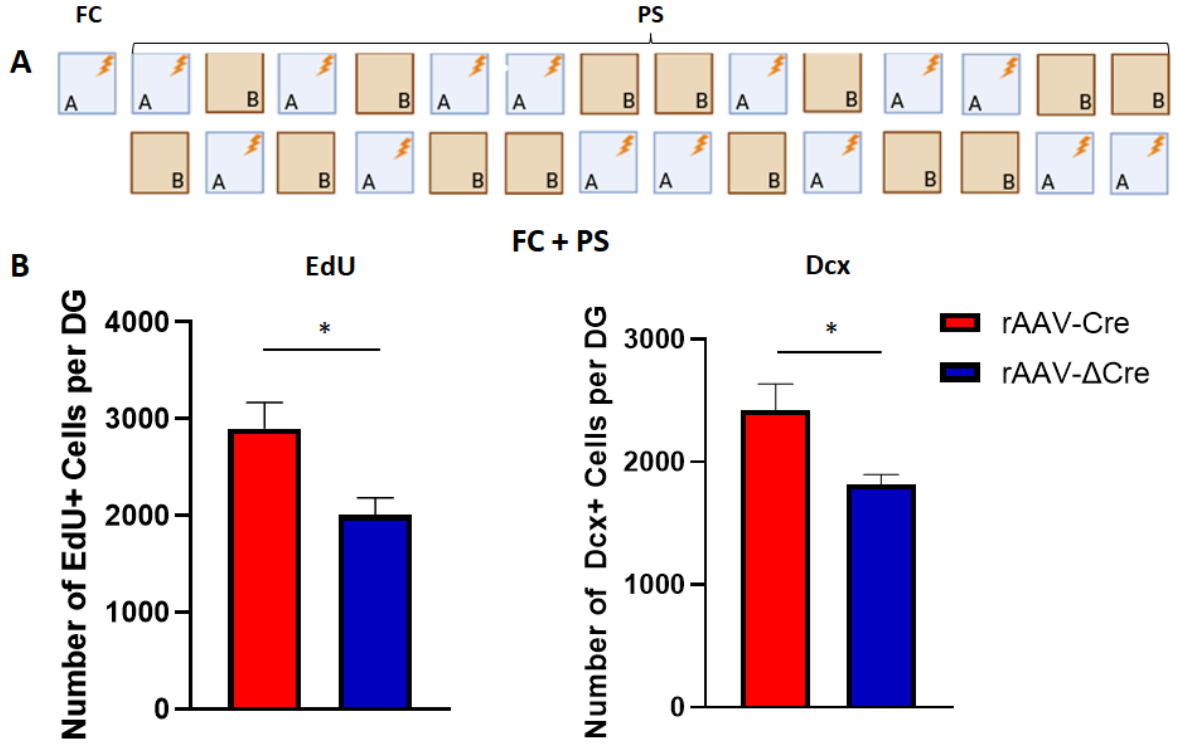

Fig (11): Elevated DG neurogenesis in mice with Cbln4 deletion that underwent pattern separation task. (A) Diagrammatic illustration of FC + PS behavioural assay undergone by the first batch of animals. (B) Findings detailing rAAV-Cre and rAAV-ΔCre mice are shown as total positive cells for EdU^+ (left) and Dcx^+ (right) per complete volume of the DG. Data is presented as mean ± SEM. Student's t-test was performed, where *$p<0.05$. (rAAV-Cre: n = 10, rAAV-ΔCre: n = 11)

We further wanted to investigate if this increased neurogenesis rate is experience-dependent or an effect of Cbln4 deletion from presynaptic inputs to the DG. To test this, we performed stereotaxic surgery to inject rAAV-Cre or rAAV-ΔCre into animals and divided them into two batches. For the first batch of animals, control and experimental groups of animals received daily EdU i.p. injections and underwent FC on day 0, but not the PS task. they were instead left in their homecages for 14 days (FC + HC). The second batch of animals received daily EdU i.p. injections for 15 days, while they did not undergo any behavioural assay. They served as the homecage-only controls, to give a measure of the basal levels of neurogenesis (HC only).

We found that among the rAAV-Cre group, there is a significant increase in the number of EdU$^+$ cells between 15d HC only group and FC + PS group (Fig. 14B; p = 0.0432) and an increase between the 15d HC only group and FC + 14d HC group, although statistically non-significant (Fig. 14B; p = 0.0515). There is no significant difference among any group of rAAV-ΔCre animals (Fig. 14A). Taken together, this suggests that AHN is dependent on the functioning of Cbln4 in conjunction with experience dependence.

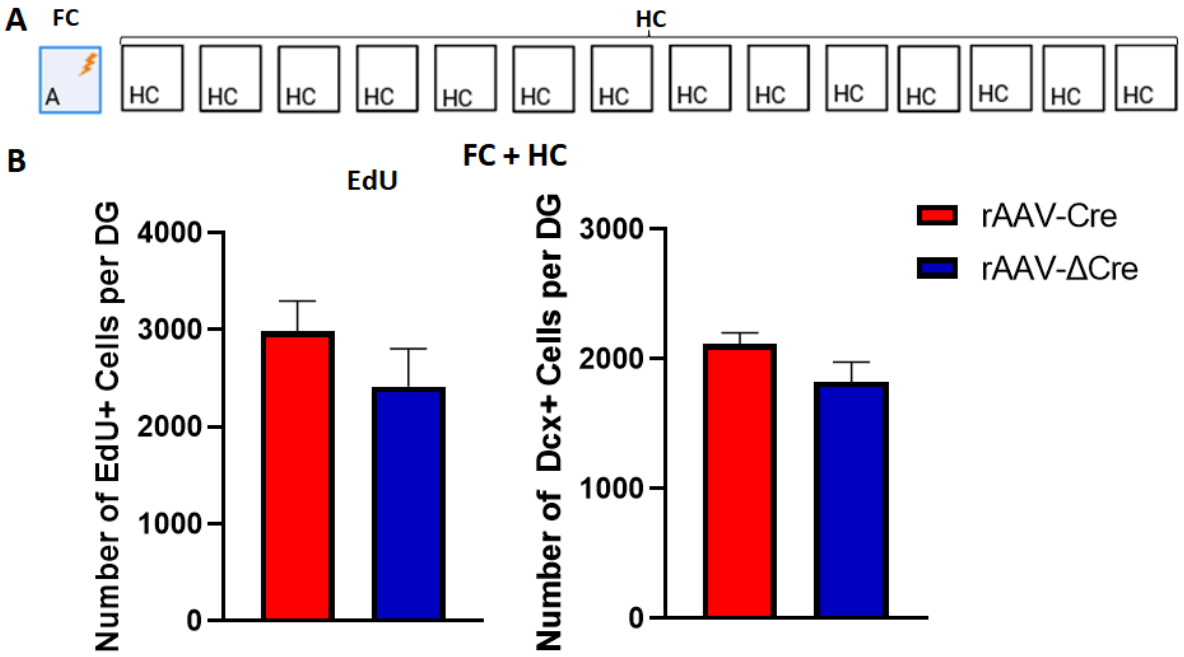

Fig (12): Neurogenesis following FC and HC for 14d (A) Diagrammatic illustration of FC + HC behavioural assay undergone. (B) Findings detailing rAAV-Cre and rAAV-ΔCre mice are displayed as total positive cells for EdU$^+$ (left) and Dcx$^+$ (right) per complete volume of the DG. Data is presented as mean ± SEM. Student's t-test was performed to test for statistical significance. rAAV-Cre: n = 5, rAAV-ΔCre: n = 4)

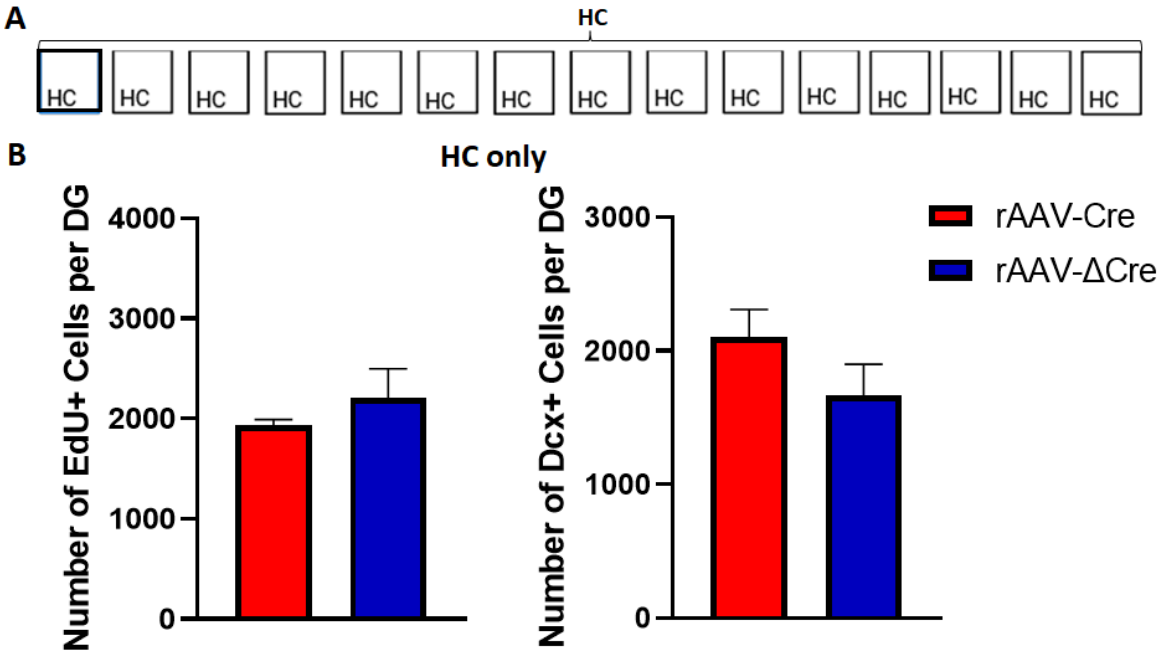

Fig (13): Steadystate monitoring of neurogenesis in HC for 15d (A) Diagrammatic illustration of FC + HC behavioural assay undergone. (B) Findings detailing rAAV-Cre and rAAV-ΔCre mice are shown as total positive cells for EdU^+ (left) and Dcx^+ (right) per complete volume of the DG. Data is presented as mean ± SEM. Student's t-test was performed to test for statistical significance. rAAV-Cre: n = 5, rAAV-ΔCre: n = 4)

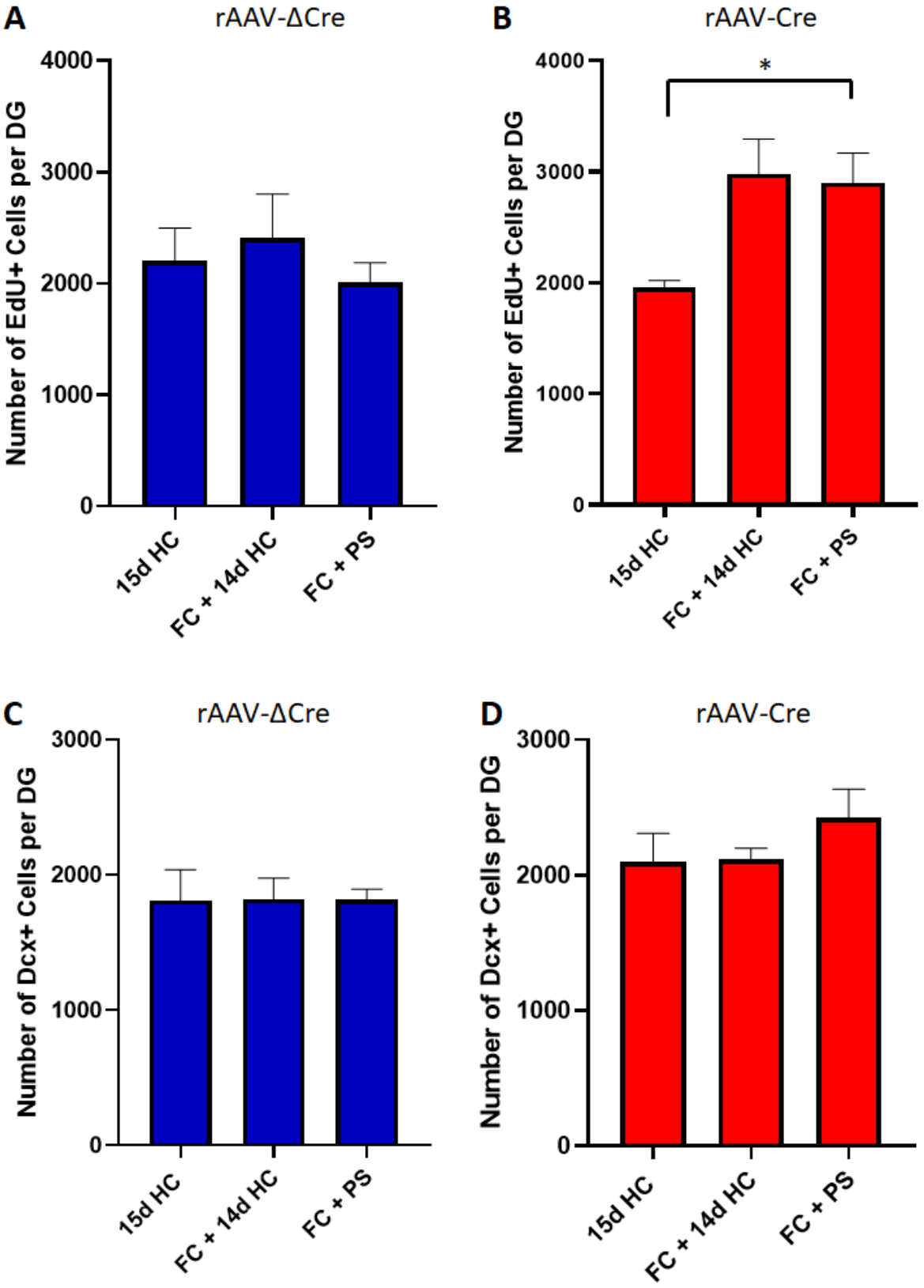

Fig (14): Experience-dependant nuerogenesis for EdU: (A) rAAV-ΔCre and (B) rAAV-Cre. Dcx: (C) rAAV-ΔCre and (D) rAAV-Cre. Data is presented as mean ± SEM. Student's t-test was performed to test for statistical significance. (FC + PS: rAAV-Cre: n = 10, rAAV-ΔCre: n = 11; FC + 14d HC: rAAV-Cre: n = 5, rAAV-ΔCre: n = 5; 15d HC only: rAAV-Cre: n = 5, rAAV-ΔCre: n = 4)

3.6 Correlation between adult neurogenesis and pattern separation

So far, our data points to pattern separation deficiency and increased AHN due to Cbln4 deletion. Next, we wanted to see if there is any correlation between AHN and pattern separation. To do this, we looked at the final neurogenesis count pertaining to EdU (Fig. 15) and Dcx (Fig. 16) of each animal and its' corresponding discrimination index on block four, when pattern separation was at its peak for both groups of animals. Here, we find no direct relationship between AHN and pattern separation for neurogenesis counts of both Edu^+ and Dcx^+.

Next, we plotted the correlation index for each group. Here, we find a slightly positive correlation for the rAAV-Cre group (Fig. 16B, 17B); however, it is not significant and the R^2 value indicates a poor fit. Therefore, from this data it is not possible to draw any conclusions about the relationship between AHN and pattern separation and how this relates to Cbln4 singalling to the DG.

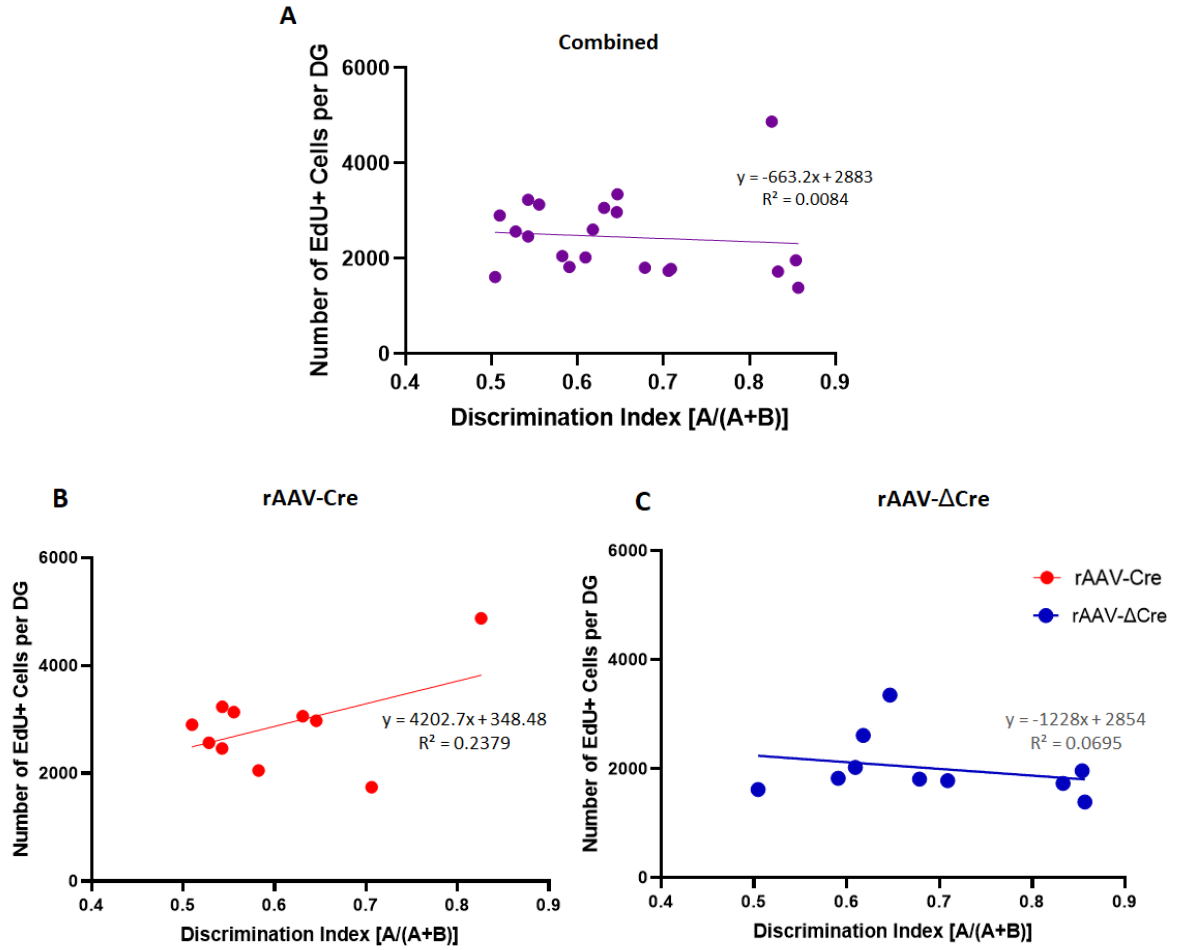

Fig (15): Correlation between total EdU^+ cells per DG and discrimination index on Block 4 (A) Both the groups of animals combined (B) Graph for rAAV-Cre and (C) Graph for rAAV-ΔCre (right).

Equation and R^2 value of linear regression are included in the graphs. (rAAV-Cre: n = 10, rAAV-ΔCre: n = 11).

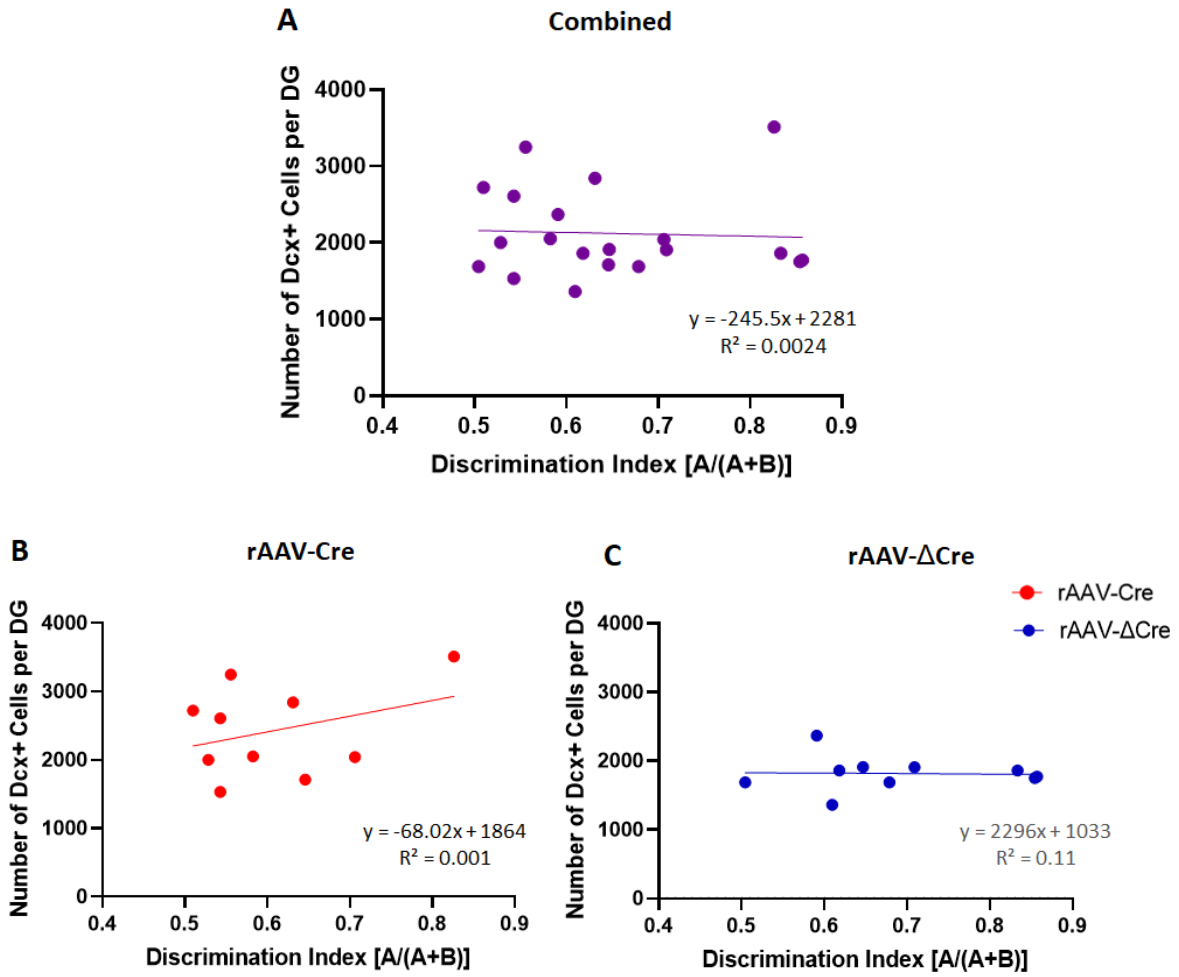

Fig (16): Correlation between total Dcx^+ cells per DG and discrimination index on Block 4 (A) Both the groups of animals combined (B) Graph for rAAV-Cre and (C) Graph for rAAV-ΔCre (right). Equation and R^2 value of linear regression are included in the graphs. (rAAV-Cre: n = 10, rAAV-ΔCre: n = 11).

3.7 Physiological properties of DG Cbln4 neurons

Next, we wanted to investigate the role of Cbln4 in basal synaptic properties of DG neurons. For this, an AAV containing either Delta cre (control) or Cre was injected into the DG of Cbln4-mVenus-tdTomato cKO mice. This selectively deleted Cbln4 from DG neurons expressing Cbln4. Two weeks later, acute hippocampal slices were prepared and whole-cell voltage-clamp method was used to measure miniature inhibitory postsynaptic current (mini IPSC) in DG granule cells. Injection sites were confirmed to have GFP expression in the DG.

A significant decrease in inter-event interval (IEI) was observed in the Cre group ($p < 0.0001$). This suggested a decrease in the overall inhibitory synaptic input of these

neurons. This could be due to changes in the number or function of inhibitory synapses.

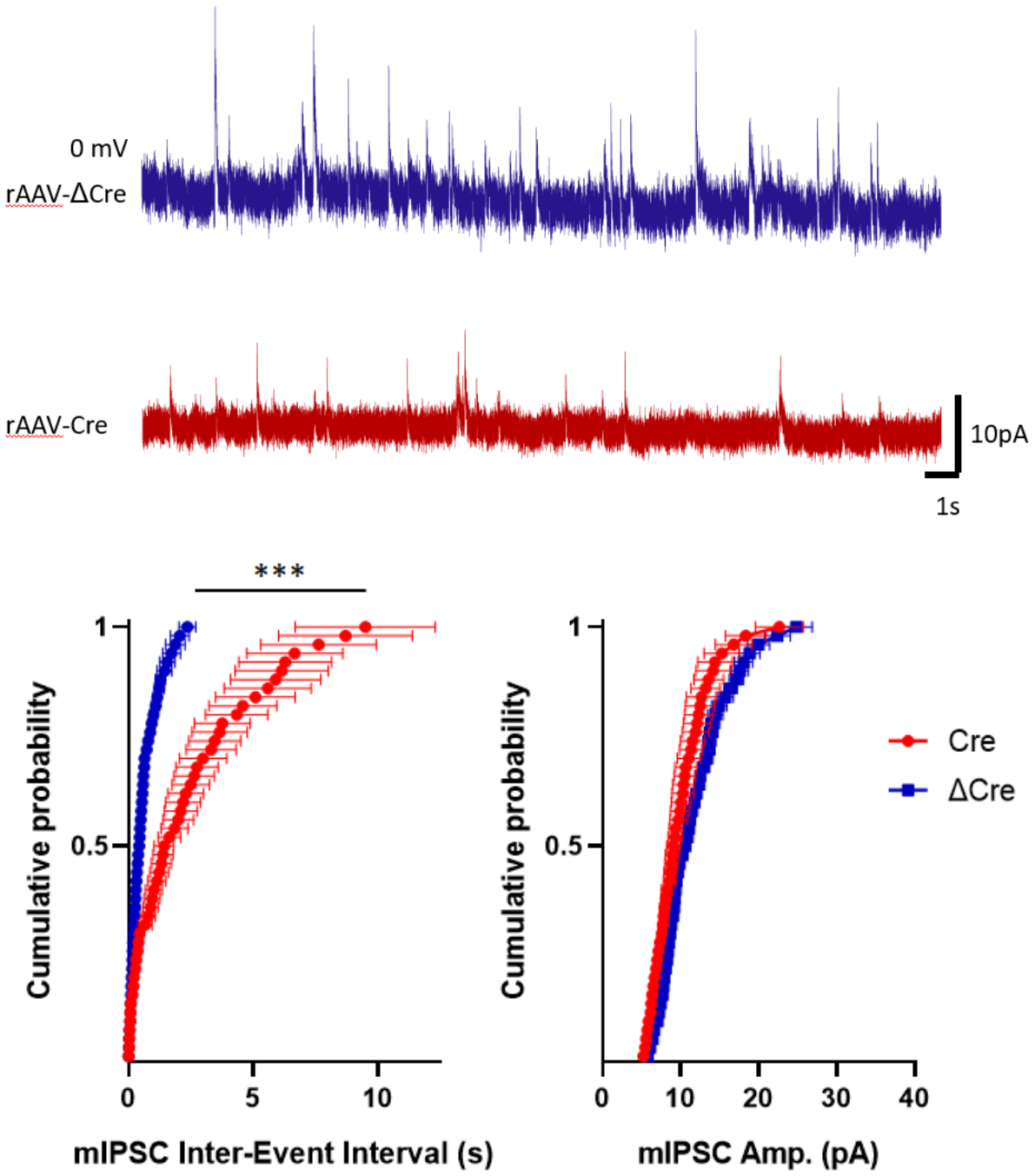

Fig (17): Top: Sample traces of mIPSC recorded at 0mV. **Bottom:** Cumulative probability curve of mIPSC inter-event intervals (Left) and Amplitude (Right). Data is presented as mean ± SEM. Kolmogorov-Smirnov test was performed to determine significant differences between the two populations (Cre vs ΔCre), where *** $p < 0.0001$ (Cre: $n = 6$, ΔCre: $n = 8$)

3.8 Role of DG Cbln4 neurons in learning and memory

We then wanted to see if changes in physiological properties of the DG granule cells could manifest a behavioural phenotype. For this, we used a contextual fear conditioning and extinction assay to test the learning and memory skills of mice injected with Cre or ΔCre.

Here, mice are subjected to contextual fear conditioning, where they receive three foot shocks and associate the fear memory to the context they are placed in. Like the pattern separation assay, freezing behaviour is subsequently monitored as a readout of fear recall. Both groups of animals display similar levels of freezing during the habituation (180 seconds before shock). They also display similar rates of learning after the first two shocks are administered but display a decrease in freezing duration after the third shock. This suggests a learning deficit due to the lack of Cbln4 in the DG.

Animals were then assessed for their memory recall on the next day. They are placed in the same context in which they received the shock, and their % freezing is quantified. Both the animals display similar freezing levels, indicating no deficit in memory recall post contextual fear conditioning. The animals were then subjected to a fear extinction paradigm, where they were placed in the fear chamber for four days without any shock being delivered. This is to disassociate the context from the fear memory, which results in a gradual decrease in freezing behaviour over the four days. Both groups of animals display similar rates of fear extinction as well. Through this, we were able to show that Cbln4 in the DG does not play a significant role in fear memory recall and extinction but might have a role in fear learning.

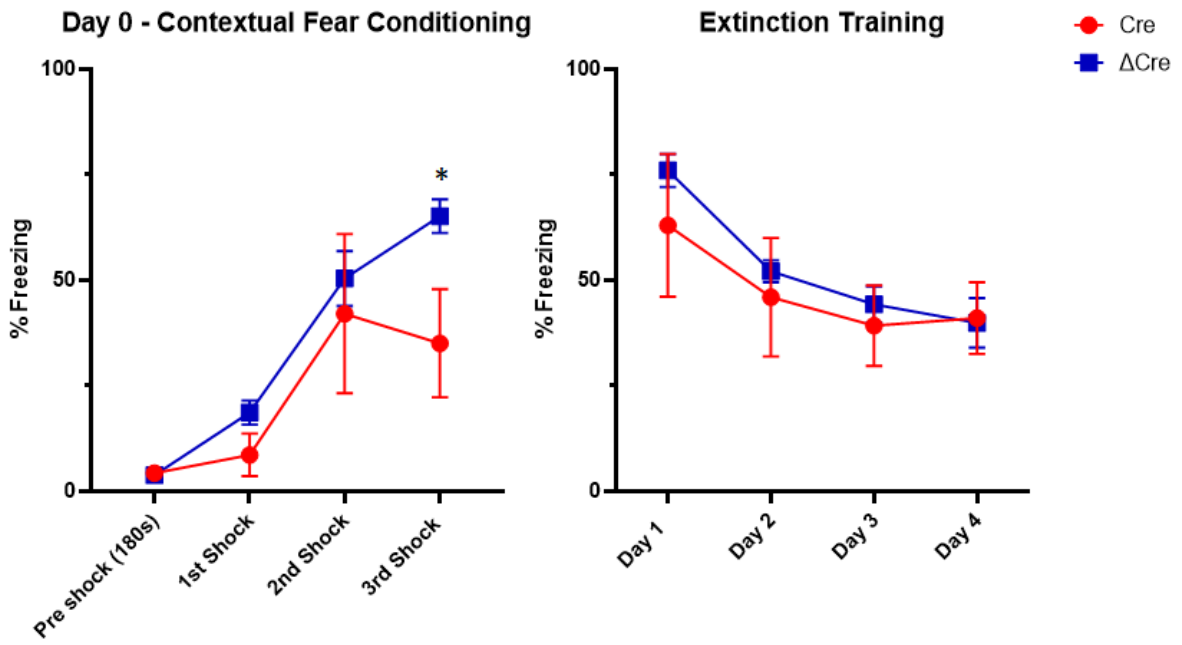

Fig (18): Contextual fear conditioning and extinction. (A) animals received three shocks in the fear chamber and were assessed for learning and memory through their freezing behaviour. (B) Animals do not receive any shock and are assessed for the extinction of their fear memory. Data is presented as mean ± SEM. Cre: n = 4; ΔCre: n = 7. Student's t-test was used to analyse the data. $p^* < 0.05$.

Chapter 4 Discussion

Adult hippocampal neurogenesis is thought to play a role in learning and memory. However, its role in memory is not clear and often controversial (Leuner at al., 2002). Several reports argue for a positive correlation between hippocampal neurogenesis and memory (Qiao et al., 2005; Jaako-Movits et al., 2005), while some studies show that neurogenesis is not necessary or often negatively correlated with memory (Dupret et al., 2008; Leuner et al., 2004; Sorrels et al., 2018). Specifically, dentate gyrus-dependent pattern separation memory has been thought to require adult neurogenesis (Sahay et al., 2011; Clelland et al., 2009), while some reports argue for a negative correlation between neurogenesis and this pattern separation (Clemenson et al., 2015).

In this project, we asked if Cbln4, a transsynaptic cell adhesion molecule that is crucial for LTP at entorhinal cortex-dentate gyrus synapses (Liakath Ali et al., 2022) as well as for pattern separation (Unpublished data), also mediates neurogenesis in the dentate gyrus. Our experiments suggest a positive regulatory role of Cbln4 in pattern separation, whereas it negatively regulates activity-dependent neurogenesis in the dentate gyrus. Our main findings are (1) Cbln4 at inputs to the DG, presumably from the EC, is necessary for pattern separation memory. (2) Contextual fear conditioning memory does not require neurogenesis or Cbln4 (3) Deletion of Cbln4 increases hippocampal neurogenesis in an activity-dependent manner during contextual fear conditioning.

Although there is an increase in newborn neurons during contextual fear conditioning in the DG of animals with Cbln4 deletion at inputs to the DG, pattern separation memory is still reduced in these animals. Along with previous data showing deletion of Cbln4 at inputs to DG ameliorates LTP (Liakath-Ali et al., 2022), our electrophysiology data indicates that Cbln4 deletion from the DG significantly reduced the frequency of mini IPSCs in granule cells (GCs).

The involvement of EC → DG circuit in pattern separation and adult hippocampal neurogenesis

A recent study from our lab has shown that EC → DG LTP is impaired in mice deficient in Cbln4 in the EC (Liakath-Ali et al., 2022). The EC → DG → CA3 → CA1 forms the hippocampal trisynaptic circuit, a critical neuronal circuit involved in memory encoding, consolidation and recall (Yau et al., 2015). The EC relays information to the hippocampus via the perforant path (PP). The PP is divided into two distinct bundles, the medial (MPP) and the lateral (LPP), based on the region of

origin, medial and lateral EC, respectively. Anatomical and behavioural studies note the distinct nature of both these paths, implying their mediation of different functions. The LPP sends olfactory, visual and auditory information to the DG, whereas the MPP transmits spatial information (Di Castro and Volterra, 2022; Moser et al., 2017; Yoganarasimha et al., 2011). Lesion studies in the MEC or LEC have shed light on distinct behavioural impairments, such as learning and memory (Hunsaker et al., 2007). Importantly, the ability to separate two patterns that are more alike than different engages the EC, and the DG (Bakker et al., 2008) was also found to be impaired in EC → DG circuit abnormalities in patients with various neurological and neuropsychiatric disorders and manipulations in rodent models (Leal and Yassa, 2018; McHugh et al., 2007; Gilbert et al., 2001).

A study has shown that stimulation of the EC increases adult neurogenesis in the DG. Subsequently, they also reported that the improvement in spatial memory of mice that had undergone this stimulation is likely due to adult-born neurons (Stone et al., 2011). Unpublished data from our lab has also shown that Cbln4 deletion from the EC results in a lack of pattern separation in these animals. Thus, this study supports this data, and we theorise that this might be attributed to the impairment of LTP in EC → DG synapses. However, a causal relationship between LTP and behavioural pattern separation is missing.

The involvement of SuM → DG circuit and other inputs in adult hippocampal neurogenesis

A recent study showed that both glutamatergic and GABAergic innervation from the hypothalamic SuM nuclei is essential for the regulation of adult hippocampal neurogenesis in the DG (Y.-D.Li et al., 2022). They found that direct SuM GABA transmission onto DG interneurons is necessary for the differentiation of neural progenitors. They also report that direct GABAergic input of SuM neurons regulates the dendritic development of adult-born neurons. In the DG, Cbln4 is primarily localised to the GABAergic hilar interneurons. Cbln4 is also highly enriched in the SuM (Seigneur and Südhof, 2017). Surprisingly, mice injected with rAAV-Cre and exposed to FC + PS over 14 days had a significantly greater number of adult-born neurons compared to animals exposed to 15d HC only. Although there is a slight increase in adult-born neurons for Cbln4 deficient mice when exposed to FC + HC for 14 days compared to the control group, the data is not statistically significant (Fig. 14B; $p = 0.0515$). There is no difference in the number of adult-born neurons injected with rAAV-ΔCre, regardless of their experiences. Altogether, this data suggests an experience-dependent regulation of neurogenesis in the DG, which might be conveyed

by the SuM, among other input regions. Future studies could focus on delineating the exact inputs involved in this curious phenomenon.

The DG also receives inputs from the Medial Septum (MS), and another study has implicated the interneurons projecting to the DG interact with quiescent neural stem cells (NSCs) through DG Paravalbumin (PV) interneurons. They also reported that the ablation of GABA neurons of the MS led to excess stem cell activation and increased adult-born neurons but consequently decreased the NSC pool size (H. Bao et al., 2017). A different study from the same lab reported the importance of PV interneurons in mediating neurogenesis in the DG (Song et al., 2013). Although Cbln4 is primarily found in the hilar interneurons of the DG, the specific subtype to which it colocalises is not known. Cbln4's presence in the MS is also not clear, but it is enriched in the lateral septum and triangular septum (Seigneur and Südhof, 2017). A previous study have also reported on the requirement of Cbln4 for GABAergic transmission in the hippocampus and for formation of new synapses in vivo and in vitro (Chacón et al., 2015). Another study reports GluD1 forms interactions at inhibitory interneurons via Cbln4 (Fossati et al., 2019). Hence, reduced GABAergic transmission from MS → DG circuit could lead to decreased adult neurogenesis and these interactions could be mediated in part by Cbln4.

Although the DG receives inputs from several parts of the brain, there is not much else known about the other presynaptic regions that could be regulating neurogenesis in the DG. For instance, the dorsal raphe nucleus (DRN) provides the dominant proportion of serotonergic innervation in the brain (Michelsen et al., 2008). It is also consistently implicated in studies related to depression (Ikuta et al., 2017). Current studies suggest that adult-born neurons are unlikely to be contributing to the etiology of depression but are probably playing a role in the behavioural effects of antidepressants (Mahar et al., 2014; Paizanis et al., 2007). It is worth noting that Cbln4 is highly enriched in the DRN (Seigneur and Südhof, 2017), and the DG → DRN circuitry could be involved in adult neurogenesis or in mediating behavioural pattern separation.

Role of Cbln4 in adult hippocampal neurogenesis and pattern separation

Although many previous studies have attributed increased adult hippocampal neurogenesis to enhanced pattern separation capabilities (Nakashiba et al., 2012; Niibori et al., 2012; Sahay et al., 2011a; Bekinschtein et al., 2011; Clelland et al., 2009; Saxe et al., 2006), our results indicate the contrary. Only one study reports a pattern separation impairment coupled with an increase in neurogenesis (Clemenson et al., 2015).

The studies mentioned previously do not describe the mechanism of action, but two major models have been proposed that could shed light on the mechanism. The first model hypothesises that "*individual neurons act cell autonomously as individual encoding neurons"* (Aimone et al., 2011). It proposes that immature adult-born neurons act as pattern integrators due to low input specificity and fine-tune their responses to subtle environmental changes.

Pattern separation is a type of neural coding where "*overlapping input patterns are coded as less overlapping output codes. One way to achieve this is via sparse coding",* and the second model suggests a regulatory role for the adult-born immature neurons in mediating this (Sahay et al., 2011b). Previous experiments have established that immature newborn neurons have a decreased threshold for firing, are insensitive to GABAergic inhibition and synapse onto the interneurons and the mossy cells in the DG hilar circuit (Gu et al., 2012; Y. Li et al., 2012; Marin-Burgin et al., 2012; Zhao et al., 2008). They propose that an increase in immature adult-born neurons increases the inhibition of mature granule cells (GC) via interneurons due to a lower threshold for firing. This results in an increase in sparse coding in GCs, which in turn improves pattern separation abilities in the DG. Our electrophysiology data indicates a decrease in inhibition of the DG granule cells due to the deletion of Cbln4 from the hilar interneurons, as observed by the decrease in mIPSC frequency, consequently increasing the activity of GCs. This could contribute to a decrease in sparse coding in the mature GCs in the DG, leading to a higher overlap of neurons firing in slightly distinct contexts, resulting in a lack of pattern separation.

Our contrasting results could be explained by utilising these above-mentioned theories - first, the increased neurogenesis could be due to a lack of Cbln4 signalling from inputs to the DG. Second, an imbalance of excitation:inhibiton ratio could lead to decreased sparse coding, consequently decreasing pattern separation ability in mice. The experience-dependent increase in neurogenesis following FC is due to the deletion of Cbln4 from the presynaptic inputs to the DG (from the Septum and the SuM), which are inputs that could regulate adult neurogenesis. Now, although this increase in neurogenesis has historically been linked to improved pattern separation abilities in mice, the EC → DG LTP impairment due to deletion of Cbln4 could explain the lack thereof in pattern separation abilities.

Theoretically, increased neurogenesis should increase sparse coding and thus improve pattern separation. However, given the reduction in inhibition of the GCs due to Cbln4 deletion from the hilar interneurons in the DG, the excitation-to-inhibition ratios of GCs favour non-specific excitation and decrease the sparse coding of the GCs, resulting in improper pattern separation. Previous studies also promote this theory

(Ikrar et al., 2013) given that somatostatin interneurons (a subtype of GABAergic hilar interneurons in the DG) are crucial for pattern separation ability in mice (Morales et al., 2020) also contain Cbln4.

Future experiments could delineate Cbln4's role in two different functions: experience-dependent neurogenesis and pattern separation. Cbln4 deletion from SuM, DRN and MS individually to investigate experience-dependent neurogenesis and a possible effect on behavioural pattern separation. Finally, in vitro, electrophysiology experiments to understand the roles of immature adult-born neurons and the deletion of Cbln4 on the network activity of DG could shed light on the exact mechanism.

We also found that Cbln4 deletion only in the DG of mice did not impair contextual memory (Fig. 16) Although these mice show slight impairment in learning, as evidenced by lower freezing duration after the delivery of the third shock, they showed no impairments in subsequent recall and extinction. This suggests that Cbln4's presence in the DG may not be necessary for learning and recall of memory but rather precise coding and pattern separation of similar cues.

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