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February 2018

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Abstract

Social recognition memory (SRM) is an essential behavior in social animals, including rodents and primates. Although the well-known engagement of olfactory inputs to hypothalamic nuclei is the primary circuit for the majority of rodent social behavior, the contribution of hippocampus to SRM remains a topic of debate. Recent results have suggested an important role of the hippocampus in SRM, with a focus on the CA2 and ventral CA1 subregions. However, the intra-hippocampal processing for SRM is not fully understood, especially the upstream input to the CA2 and CA1 subregions, the CA3 and dentate gyrus (DG). To address the potential contribution of the CA3 and the DG to SRM, I tested SRM in mice with the NMDA receptor subunit 1 gene (NR1) deleted specifically in CA3 and DG principle cells. Mice with NR1 knockout (KO) in CA3 pyramidal cells demonstrated impaired SRM; however, mice lacking the same gene in DG granule cells performed indistinguishably from littermate controls. Moreover, with acute manipulation of CA3 activity by designer receptor exclusive activated by designer drug (DREADDs), I found that ventral but not dorsal CA3 is required for the encoding of SRM. Together, my results strengthen the idea that hippocampus is involved in SRM in mice, and further suggest that ventral CA3 is necessary for memory encoding.

Chapter 1 Introduction

Social behavior and social memory

A variety of behaviors constitute animals' daily lives, such as foraging, sleeping, communicating and mating. Social behaviors can be defined as sets of activities involving interactions between individuals of the same species that influence immediate or future behaviors[1], such as social motivation, social recognition, social interaction, aggressive interaction and integration[2]. One fundamental question is if animals have social memory and how or if that social memory affects social behavior? Evidence supporting the existence of social memory in rodents and its impact on social behavior is the monogamy of prairie vole (*Microtus ochrogaster*)[3]. These animals have long-lasting partnerships, demonstrate aggressive behavior toward strangers and take care of their offspring[4]. The other type of social memory, termed social recognition memory (SRM), is more general. In SRM animals recognize individual conspecifics. In humans, we remember our family, siblings and friends and recognize strangers, and the integrity of SRM can be determined by language report. In rodents SRM research, SRM integrity is measured by observing their innate behavior, in which the animal shows higher propensity of social interactions, including sniffing, following and adjacent lying, toward novel individuals and reduces the behavior intensity after familiarization. Indeed, the test for social recognition memory usually consist of two sessions of interaction, sampling (encoding) and recognition (retrieval). For familiar recognition, animals demonstrate a significant reduction of investigation; whereas for novelty recognition, the investigation time in both sessions were comparable[5]. The following question is then what kind of information are encoded for social recognition memory? In human's case, we remember a person's face (visual input), vocal trait (auditory input), and sometimes smells (olfactory input). In rodents, it is

believed that social information mainly transmit through pheromones and can be perceived by a special olfactory organ, called the vomeronasal organ (VNO)[6]. At a neural circuit level, it has been shown this olfactory information can be transduced to the entorhinal cortex[7], amygdala[8], hippocampus, and to the hypothalamus to drive proper behavioral responses. Research also indicated that Olfacto-Hippocampal network is involved in odor-discrimination learning[9]. However, whether the hippocampus participates in social recognition memory still remains controversial.

Social memory and hippocampus

The first experiment to examine the role of the rodent hippocampus in social memory could be traced back to Kogan's research nearly 20 years ago[10]. In their work, mice with hippocampal lesion exhibited severe social recognition impairments, unable to recognize a familiar juvenile mouse. While interesting, their work did not address which region or circuit within the hippocampus was related to the behavioral changes. However, in Bannerman et al., 2001 they showed that rats with hippocampal lesion did not demonstrate impaired social recognition memory[11]. These two studies raised the debate whether the hippocampus is involved in social recognition memory. Recently, with the progress in the genetic manipulation, more precise hippocampal subregions targeting has clarified the situation. Two studies have implicated hippocampal region CA2 as an essential component of the social memory circuit[12, 13] and one follow up work further indicates the possible mechanism of hippocampal CA2 for social memory representation[14]. Moreover, with the activity-dependent labeling and optogenetic manipulation, Okuyama et al., 2016 also demonstrated pyramidal cells in ventral CA1 store social memory[15]. These studies support the idea that hippocampus is involved in social

recognition memory. The following question is that what the neural mechanism of hippocampus underlying the social memory. Before touching this question, it is necessary to know the fundamental properties of hippocampus in learning and memory.

Hippocampus and Learning & Memory

The most fascinating characteristics of brain function are **learning** and **memory**. Learning refers the process of new information being acquired by the nervous system and can be observed by the changes in behavior. Memory represents the **encoding**, **storage** and **retrieval** of learned information by the brain. Memory is not a uniform phenomenon. First, memory can be described by a temporal aspect, short-term memory versus long-term memory; the former type of memory exists form few seconds to minutes, the latter type of memory stays for days, months to years. Long-term memory can be further categorized into two major types, implicit memory and explicit memory. Implicit memory usually is shown by behavior execution, including skill learning, priming and conditioning. Explicit memory represents things you know that you can tell others, usually episodic and semantic. Back to 1950s, the first case which indicates the necessity of hippocampus for episodic memory formation has been revealed by the most famous case report form patient H.M., Henry Gustav Molaison (1926-2008). To alleviate his severe epilepsy, Dr. Scoville removed his medial temporal lobes bilaterally, including the hippocampi, parahippocampal cortices, entorhinal cortices, piriform cortices and amygdala. H.M. successfully recovered from the epilepsy but surprisingly demonstrated a severe anterograde amnesia, resulting in an inability to encode new information to form long-term memory after surgery[16]. To further specify the amnesic symptom in Henry, different groups of researchers had done intensive tests on him and determined that his episodic memory was exclusively impaired while his semantic memory and implicit memory were intact. According to his as well as 9 other amnesic patient cases, which have suggested that the hippocampus is required for human episodic memory, the so called “who, what, where, when” memories which

define our daily lives.

We learned that the hippocampus is crucial for episodic memory. The hippocampus is located in the medial temporal lobe of the brain, which shows structural conservation across different species, including rodents and humans. The anatomical structure of hippocampus comprises of the following subregions, CA1, CA2, CA3, and dentate gyrus (DG)[17](**Fig.1**). The CA4 region

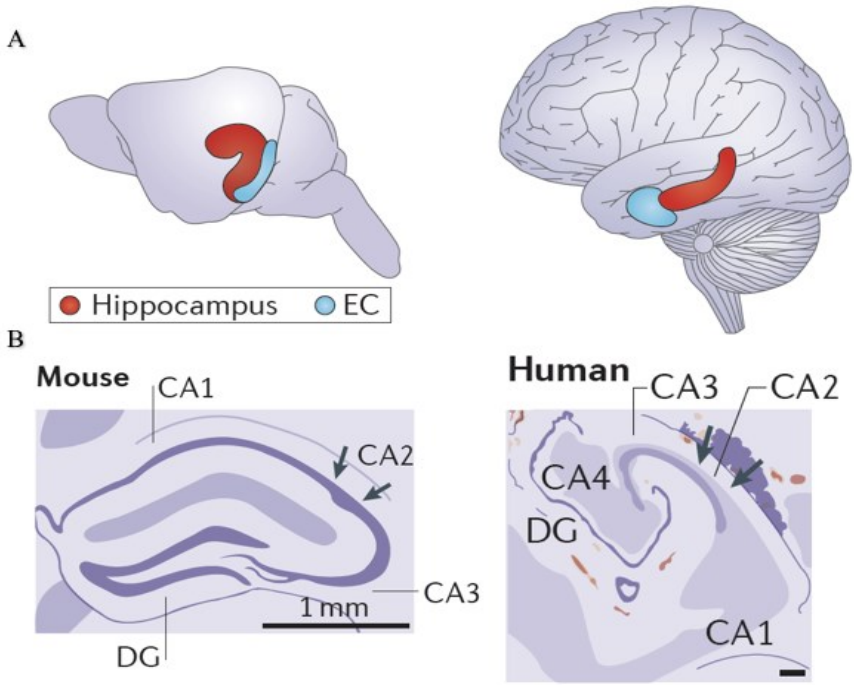


Figure 1. Hippocampus Cross-species Comparison. **A**. The long axis of hippocampus (red) in rats and humans with the adjacent entorhinal cortex (EC, shown in blue). **B**. Drawing of Nissl sections form house and human hippocampus. Adapted by permission from [**Springer Nature Terms and Conditions for RightsLink Permissions Springer Customer Service Centre GmbH**]: [**Springer Nature**] [**Nature Reviews Neuroscience**] [**Functional organization of the hippocampal longitudinal axis**, **Bryan A. Strange, Menno P. Witter, Ed S. Lein, Edvard I. Moser**), [**2014**]

was first defined by Lorente de No in 1930s, but later clarified by Theodor Blackstad (1956) and David Amaral (1978). This CA4 region is actually the deep, polymorphic layer of the DG, called hilus which contains the mossy cells[18]. The classical depiction of hippocampal

connectivity is called the trisynaptic loop, and best studied in the rodent system. In this circuit, the information arrives to entorhinal cortex (EC) and then is relayed to DG through the perforant path. From the DG, the information flows to CA3 through the mossy fiber (MF) and finally CA3 projects via the Schaffer collaterals to CA1. The processed information is then output to subiculum and back to EC (**Fig. 2**)[19]. Recent work has demonstrated that the largely ignored hippocampal subregion CA2 has distinct functionality[20-24] and connectivity[20, 25, 26].

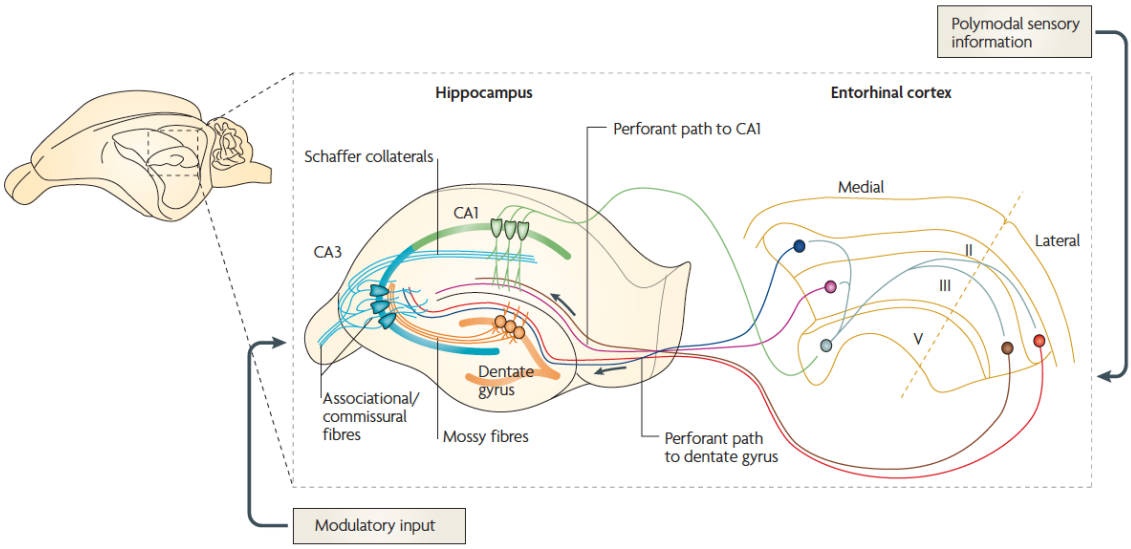


Figure 2. Anatomy of the rodent hippocampus. Adapted by permission from [**Springer Nature Terms and Conditions for RightsLink Permissions Springer Customer Service Centre GmbH**]: [**Springer Nature**] [**Nature Reviews Neuroscience**] [**Synaptic plasticity, memory and the hippocampus: a neural network approach to causality**, **Guilherme Neves, Sam F. Cooke, Tim V. P. Bliss**), [**2008**]

In fact, the studies of hippocampus in humans and in rodents have been largely separated[27]. Researches in human have focused on the episodic memory perspective; however, in rodent, researchers have more focused on the spatial learning and navigation, which is based on the discovery of 'place cells' in rat hippocampus in 1971. The place cell properties have been linked to the spatial memory and the establishment of a cognitive map[28] of the world in rodent's brain.

For hippocampus-dependent memory formation and behavioral execution, it is well accepted that information undergoes three phases of processing, encoding, consolidation and retrieval; each phase requires the involvement of distinct hippocampal subregion[20]. During information encoding, DG activity is essential for the pattern separation[29], the ability to distinguish the distinct characteristics of each input; CA3 plasticity is required for fast encoding[30-32] and its transmission is crucial for accurate CA1 temporal coding[33]. CA3 recurrent networks are thought to be important for the off-line memory consolidation[34]. For memory recall, CA3 recurrent collaterals mediate pattern completion[35-37] and modulate CA1 activity by Schaffer collateral. In the episodic memory view of hippocampus, researchers further advanced the idea by providing processing mechanisms across species and across tasks[27]. Many studies have suggested the important role of hippocampal CA3 in regulation of CA1 activity. In the following sections, I'd like to discuss in more detail of CA3 network and its function.

Local circuit of hippocampal CA3

CA3 has several characteristics that distinguish it from the other hippocampal subregions. Under the local circuit perspective[38, 39] (**Fig. 3**), CA3 pyramidal cells receive three excitatory

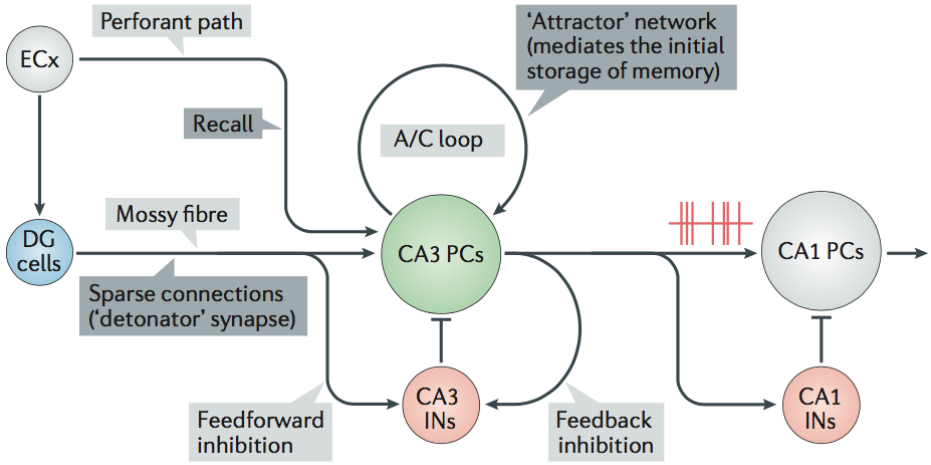


Figure 3. CA3 local circuit. Adapted by permission from [**Springer Nature Terms and Conditions for RightsLink Permissions Springer Customer Service Centre GmbH**]: [**Springer Nature**] [**Nature Reviews Neuroscience**] [**Operation and plasticity of hippocampal CA3 circuits: implications for memory encoding**, **Nelson Rebola, Mario Carta, Christophe Mulle**), [**2017**]

inputs: **First**, the dentate gyrus sends sparse but powerful axons to the proximal dendrites of CA3 pyramidal cells through mossy fibers. Although sparse, a single granule cell can depolarize the CA3 network reliably[40]. These sparse but powerful synapses are also called **detonator** synapses. **Second**, the entorhinal cortex, especially the lateral entorhinal cortex, sends axons onto the outer half of the stratum lacunosum-moleculare (slm) of CA3 apical dendrites through the perforant path. It has been suggested that the input from the performant path operates as a pattern associator to initiate retrieval[41]. **Third**, the CA3 pyramidal cells send axons to CA3 pyramidal cells ipsilaterally and contralaterally through associational fiber (also called recurrent collaterals, A/C loop) and commissural fibers, respectively. This is the most

unique characteristic of intra-hippocampal connection. This autoassociational connections are thought to work as **attractor** network, which maintains a stable state within the network. It has also been proposed that each CA3 pyramidal cell receives approximate 46 mossy fiber inputs, 3600 perfroant path inputs and 12000 recurrent collaterals[42]. Based on the theories have suggested a crucial role of the CA3 network in forming memories. Any new event to be memorized is represented as a CA3 firing pattern, which is triggered by mossy fiber input and is maintained by the recurrent collateral connections. During memory retrieval, each pattern can be fully reactivated when the information of retrieval cue was provided by the perforant path firing. In the past hippocampal subregions were considered as homogenous units; therefore, it is worth to understand whether the hippocampus acts in unity or has distinct function domain. The short answer is that the hippocampus has functional organization along the longitudinal (also known as dorsal-ventral) axis. In the following section, I will discuss the dorsal-ventral difference of hippocampus, especially in the CA3 subregion.

Dorsal and ventral difference of hippocampal CA3

Anatomical data have suggested that the recurrent collaterals of CA3 project differently across the transverse (proximal/distal) plane and longitudinal (dorsal/ventral) plane[43]. The proximal CA3 (which is close to DG, also noted as CA3c) preferentially projects to dorsal CA3, the mid portion (also noted as CA3b) projects equally to both dorsal and ventral CA3, and distal CA3 (which is close to CA1, also noted as CA3a) preferentially projects to ventral CA3[38]. The projection from CA3 to CA1 is called the Schaffer collaterals, and the overall axons terminate in stratum radiatum and stratum oriens of CA1. The anatomical evidence also suggests that the proxiamal CA3 tends to project to distal CA1, which is close to subiculum; whereas the distal CA3 projects to proximal CA1, which is close to CA2. Moreover, at the dorsal level, the

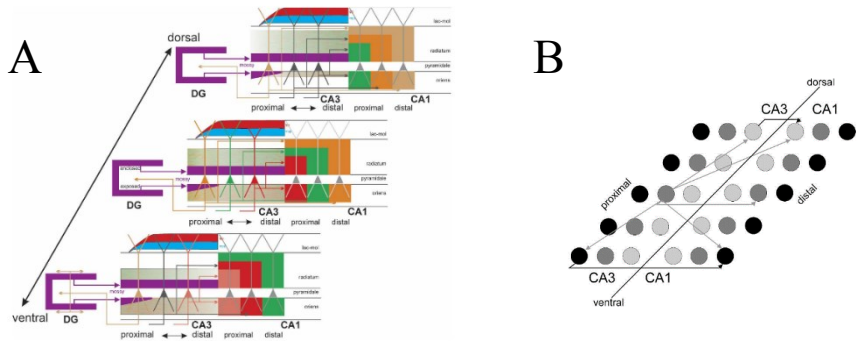


Fig. 4 Intrinsic and extrinsic wiring of CA3- Indications for connectional heterogeneity. (A). The neural connection across dorsal/ventral axis and transverse (proximal/distal) axis. (B). Simplified CA3 connectivity in dorsal/ventral and transverse view. Adapted by permission from Creative Commons Attribution-Non-Commercial 4.0 International License (CC-BY-NC) [**Intrinsic and extrinsic wiring of CA3: Indications for connectional heterogeneity, Menno P. Witter, 2007]**

CA3 collaterals are located in the deep layer of CA1 stratum radiatum and oriens; however, at more ventral level, the CA3 collaterals are located in more superficial layer of CA1 stratum radiatum and less in stratum oriens. Besides, the dorsal-ventral projection preference is also

found in CA1, the dorsal CA3 projects to dorsal CA1, ventral CA3 projects to ventral CA1, and the intermediate CA3 projects to both dorsal and ventral CA1[38]. The intrahippocampal connections are graphically summarized in figure 4. Moreover, recent advanced progress in molecular techniques has further addressed and deciphered the difference between dorsal and ventral hippocampal of its gene expression as shown in figure 5. Based on the boundaries of selective gene expression, the CA3 subregion can be split in to 9 subdivisions, according to the proximal/distal and dorsal/ventral location[44, 45]. These distinct genomic expression and

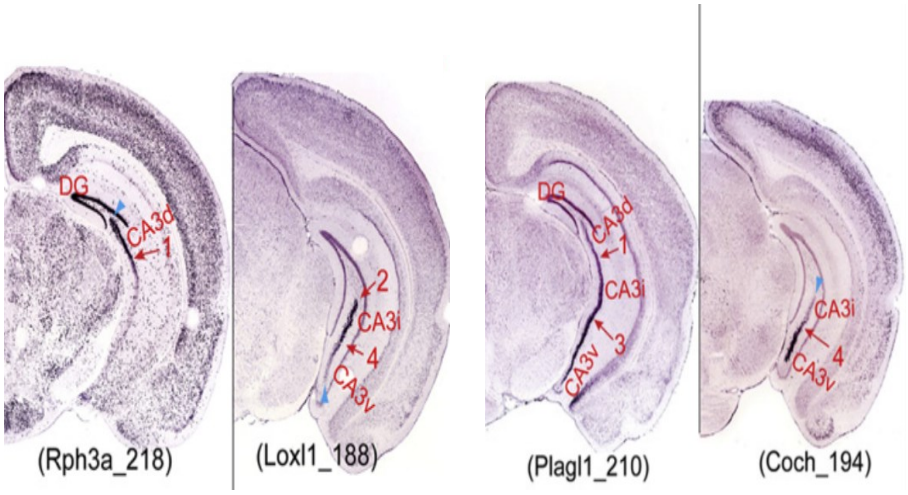


Fig. 5 Gene expression in CA3 showed regional preference across dorsal-ventral axis. Adapted from Neuron 65/1, Michael S. Fanselow, Hong-Wei Dong, Are the Dorsal and Ventral Hippocampus Functionally Distinct Structures? Pages No. 13, Copyright (2010), with permission from Elsevier

anatomical connections across dorsal-ventral axis raises the question of whether the dorsal and ventral hippocampus CA3 have different physiological properties during memory processing. Although there exists a huge body of research on hippocampal physiology following the discovery of place cell activity in 1970s, the major findings in hippocampal activity were focused on CA1 and the dorsal hippocampus. Work comparing the dorsal-ventral difference of CA3 is limited. To date, only two studies systematically compare the dorsal-ventral properties of CA3. The first report in 2008 found that the ventral CA3 also had place fields and the size

of the place fields gradually increased from dCA3 to vCA3[46]. A study in 2010 have further extended the physiological properties of vCA3 in comparison with dCA3. The vCA3 cells showed larger spatial coverage, lower spatial information content, lower firing stability, lower theta power and theta rhythmicity[47]. These physiological data have suggested the different information processing along the dorsal-ventral CA3. The emerging question then be what kind of behavior tasks require hippocampal CA3?

Behavioral regulation of hippocampal CA3

To discuss the contribution of hippocampal CA3 to animal behavior, it is important to recall that hippocampal CA3 has two major outputs, one is to CA3 area through recurrent collaterals and the other one is to CA1 area through the Schaffer collaterals. I first will introduce the necessity of CA3 recurrent collaterals for behavior. Pharmacological blockade of NMDA receptor[48], cytotoxic lesions[49] and the NR1 knockout[30] in CA3 all lead to a similar behavior deficit, the animals are unable to perform **rapid encoding** of novel information. It has also been demonstrated that CA3 is essential for **multiple trial encoding**. Lesions of CA3 caused the object-place and odor-place association learning deficits[50], tasks which in animals require multiple trials to acquire the behavior. One of the most distinct function of CA3 is **pattern completion**, by which the animals can retrieve the complete memory by only receiving partial cues. This function depends on the NMDA plasticity in CA3. Mice with a CA3 specific NR1 knockout could perform the standard water maze task; however, under partial cue removal, the animals showed behavior impairments[51]. Direct neurophysiological evidence supporting CA3 for pattern completion was introduced by Joshua Neunuebel, in 2014. In their local-global reference frame test of the CA3 produced output patterns resemble to the original representation in the local-global cue mismatched environment[35]. These research evidences has suggested the essential role of CA3 recurrent collaterals for animal behavior.

The other major output of CA3 are the Schaffer collaterals, which provide a feed-forward regulation of CA1 information processing. Extensive studies have suggested that CA1 has at least four important functions, including temporal processing, association across time, intermediate memory, and consolidation of new information[42]. From the CA3 lesion studies,

the behavioral impairments were found in spatial location and odor temporal order learning, but not in the intermediate memory and consolidation, which indicate there are two modes of information processing between CA3 and CA1. The **parallel processing** suggests that the perforant path provides main input into CA1 for an intact behavioral performance, while the **interactional processing** suggests the CA3 feed-forward Schaffer collateral input to CA1 modulates behavioral performance. The contributions and theories of two CA3 projections for animal behaviors have extensively reviewed by Raymond P. Kesner[42, 52].

In addition to the contribution of hippocampus to normal behavior, in the pathophysiological perspective, there are also evidence suggesting the relationship of hippocampal malfunction to psychiatric disorders, including temporal lobe epilepsy (TLE)[53], the presence of severe seizures, and schizophrenia. Pathological data have indicated that the dysfunction of hippocampal CA2 might be involved in schizophrenia, which is well reviewed recently[54]. Patients with temporal lobe epilepsy showed cell loss predominantly in CA4, and partially in CA3 and DG. Animal models for TLE have identified the basic cellular mechanisms of epileptogenesis, one of the most useful protocol for acute seizures induction is to introduce excitatory neurotoxin kainic acid (KA) into animals. Our previous research has demonstrated the transmission of hippocampal CA3 is crucial for KA-induced seizure because of the rich expression of KA receptors and its strong recurrent network. Blocking the CA3 transmission by the expression of the tetanus toxin (TeTX) can attenuates KA-induced seizure[55].

In summary, I have introduced the idea of social memory, the involvement of hippocampus in social memory, and the underlying circuit connection and mechanism for hippocampal-dependent learning memory. In addition, the research on CA2 and ventral CA1 have shown that

these subregions are important for social recognition memory. However, regarding the reciprocal modulation between CA2 and CA3[24] as well as the intra-hippocampal connection from vCA3 to vCA1[38], the social recognition memory impairment might not simply because of dysregulation of a single hippocampal subregion. Information processing by CA3 and DG may also important for social recognition memory. Therefore, a comprehensive comparison of the contribution of each hippocampal subregion for social recognition memory is needed, especially the previously ignored CA3 and DG.

To dissect the contribution of CA3 and DG to social recognition memory here I take advantage of conditional genetic access to manipulate synaptic plasticity and excitability in a subregion specific manner. I find that a loss of NMDA receptor dependent synaptic plasticity in all CA1 or CA3 pyramidal cells, but not in the granule cells of the DG, impairs social memory. Furthermore, acute inactivating CA3 activity specifically in the dorsal or ventral regions demonstrate that the dorsal CA3 activity seems dispensable for social recognition memory, however, the ventral CA3 activity is essential for social recognition memory during the encoding phase of the task. My data not only demonstrate the involvement of CA3 in social recognition memory, but also clarify a larger social circuit across the multiple axes of the hippocampus and further determine the potential phase for social memory processing.

Chapter 2 Materials & Methods

Animals

By using advanced molecular biological approaches, overexpression or knockout of a specific gene in a specific population of cells can be achieved. In my research, I applied five different genetic manipulated mice for experiments, including three region-specific (CA1, CA3 and DG) NR1-KO mice, CA3-TeTX (tetanus toxin, TeTX; a VAMP2 protease which blocks vesicle docking and prevents neurotransmitter release) transgenic mice and CA3-Cre transgenic mice. The characteristics of each manipulation is introduced below. Generation of hippocampal subregion specific NR1-KO mice requires the breeding of region specific Cre transgenic mice to the floxed-NR1 (*f*NR1) transgenic mice[56]. I will introduce the region-specific Cre transgenic mice first and then the region specific NR1 KO mice and CA3-TeTX transgenic mice in the following section.

CA1-Cre transgenic mice

The CA1-Cre transgenic mouse line (CW2) was generated in the Tonagewa lab by Chanel Lovett. The homozygous *cre* epression is driven by the αCaMKII promoter in C57BL/6 background mouse. The construct used to generate CW2 line was identical to the T29-1 line which was used to generate CA1-NR1 KO mice[56].

CA3-Cre transgenic mice

CA3-Cre transgenic mice were first introduced by Nakazawa et al in 2003[30]. The homozygous *cre* expression is driven by the kainate receptor subunit KA-1(*Grik4*) promoter. The expression specificity was examined by crossing this KA1-cre line with a *lacZ* reporter line and by immunohistochemistry. The *Cre/loxP* recombination can be detected in nearly 100% of CA3

pyramidal cells, around 10% of DG and cerebellar granule cells, but not in GABAergic interneurons.

DG-Cre transgenic mice

The DG specific cre expression was originally established by Balthasar in 2004[57], in which the *cre* transgene expression is driven by proopiomelanocortin (POMC) regulatory elements, and later been demonstrated a robust Cre/loxP recombination in DG granule cell layer across dorsal/ventral axis[29].

CA1-NR1 KO mice

CA1-NR1 KO mice were generated by crossing CW2 mice with *fNR1* mice. Most of the *in vivo* physiological properties, such as firing rate and complex spike index, were indistinguishable between controls and KO mice[58]; however, these KO mice showed impairment in spatial representation and spatial memory[56, 58].

CA3- NR1 KO mice

CA3-NR1 KO mice were first introduced along with the CA3-Cre mice, which were generated by crossing CA3-Cre mice to *fNR1* mice. The gene is nearly completely deleted by 18 weeks. In the CA3-NR1 knockout animals, which had normal spatial memory encoding and retrieval in the Morris water maze test; however, they were impaired in retrieving the memory with the incomplete cue presentation[59].

DG-NR1 KO mice

DG-NR1 KO mice were generated by crossing POMC-cre mice with *fNR1* mice, where the gene and protein were nearly absent by the age of 16-weeks. These KO animals learnt the Morris water maze task and contextual fear conditioning task; however, they cannot distinguish

the similar but distinct contexts in a modified contextual fear conditioning[29].

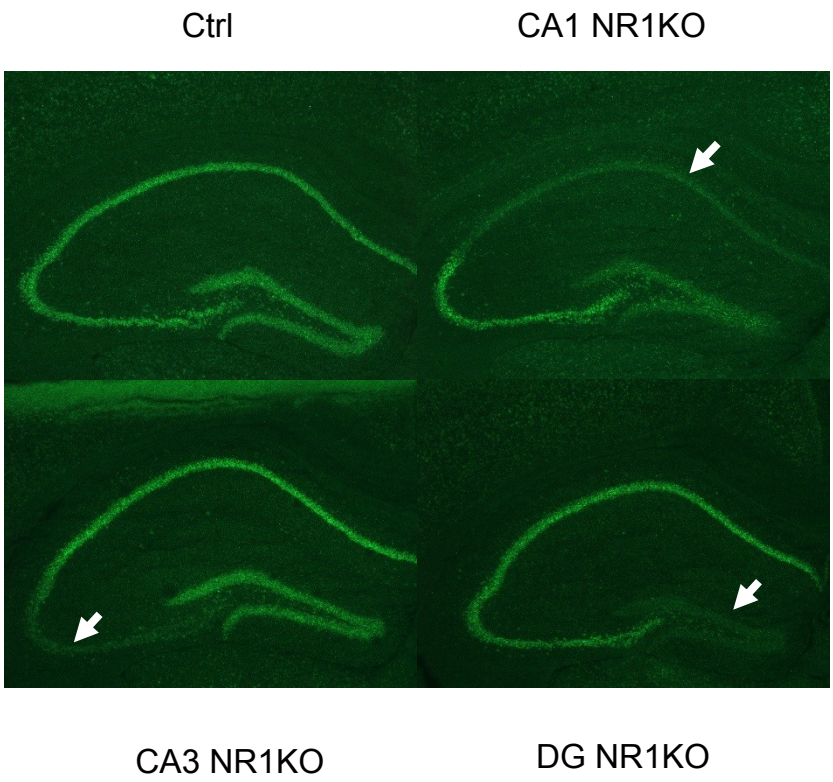


Figure 6. *NR1* mRNA *in situ* hybridization. White arrow indicates site of gene deletion. Chiang, M. C. *et al*., Behav Brain Res. 2018. ***In press***

Examination of *NR1* deletion was examined by *in situ* hybridization (**Fig. 6**) performed by Marie E Wintzer in McHugh Lab which showed comparable results with previous study[60].

CA3-TeTX transgenic mice

CA3-TeTX mice were first introduced by Nakashiba et al., 2008[32]. In brief, the CA3-TeTX mice were generated by a doxycycline (DOX)-inhibited circuit exocytosis knockdown (DICE-K) system, which crossed from three transgenic mice, the CA3-Cre mice, the αCaMKII-tTA mice and the TRE-TeTX mice. VAMP-2 staining demonstrated the CA3 to CA1 transmission was block after 4 weeks DOX withdrawal (**Fig. 7**). Transgenic expression of tetanus toxin, which is specific to CA3 pyramidal cells and allows chronic shut-off of CA3 neurotransmission[34]. Moreover, TeTX expression is driven by TRE promoter, which is

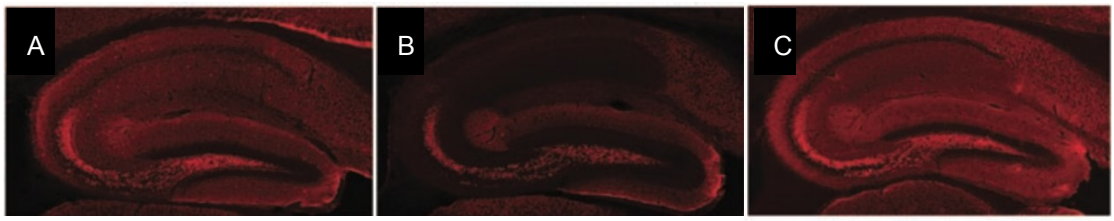


Figure 7. VAMP-2 staining of hippocampal sections from CA3-TeTX mouse. (A) CA3-TeTX animal raised on DOX food, (B) 4 weeks DOX withdrawal and (C) 3 week DOX withdrawal followed by 7 weeks DOX readministration. From Nakashiba, T., Young, J.Z., McHugh, T.J., Buhl, D.L. & Tonegawa, S. Transgenic inhibition of synaptic transmission reveals role of CA3 output in hippocampal learning. *Science* **319**, 1260-1264 (2008). Reprinted with permission from AAAS.

controlled by the induction of tetracycline or the related compound, doxycycline, in the food or water of the animal. By providing food with doxycycline to mice, the expression of TeTX was suppressed; while removing doxycycline from food for three weeks, the expression of TeTX can reach plateau and block neurotransmission. In addition, the CA3 transmission is also been demonstrated indispensable for fast encoding, pattern completion-based memory retrieval[32] and contextual fear memory consolidation[34].

All of the subjects were group housed after weaning were maintained in a temperature- and humidity-controlled room with a 12 h light/dark cycle (lights on from 8:00 A.M. to 8:00 P.M.). The food and water were accessed *ad libitum*. All experimental protocols were approved by the RIKEN Institutional Animal Care and Use Committee.

Adeno-associated viruses (AAV) vectors

The adeno-associated virus (AAV) vectors pAAV.synapsin.DIO.hM4D(Gi).mCherry (I term hM4D(Gi) hereafter) and pAAV.EF1a.DIO.mCherry (I termed mCherry hereafter) were generated in our laboratory. Each AAV vector was co-transfected with pAAV-DJ/8 and pHelper into the 293FT cell line (Invitrogen) using the 293fectin transfection reagent (Invitrogen). After 72 hours, the supernatant was collected and centrifuged at 3,000 rpm for 30min and then filtered

through a 0.45μm filtration units. Purification of AAV was achieved by ultracentrifugation (87,000 g, 4°C, 2h) with 20% sucrose cushion. After the ultracentrifugation, supernatant was discarded and the pellet was resuspended in phosphate-buffered saline (PBS), aliquoted and stored in -80°C for long-term storage.

Surgery for virus stereotaxic injection

CA3-Cre mice were anesthetized by using Avertin (2, 2, 2-Tribromoethanol; Sigma-Alderich, Japan, intraperitoneal injection, 0.5mg/g), and placed into stereotatic frame (Narishige, Japan). A micro syringe (World Precision Instrument; Nanofil 07E, USA) and the needle (World Precision Instrument; NF35BL-2, USA) were filled with virus. Viruses were bilaterally injected into dorsal CA3 (coordinates from bregma: AP: -1.7mm; ML: ±2.5mm; DV: -2.0mm) or ventral CA3 (coordinates from bregma: AP: -3.0mm; ML: ±2.5mm; DV: -4.5mm) with the following parameters: 500nl/hemisphere; injection speed: 200 nl/min; post-injection waiting: 5 min. All mice were given 2 weeks for post-injection recovery and virus expression before the behavior test.

Social memory test paradigm

Mice were habituated to the test environment for 30min prior to the test. The direct interaction test is comprised of two sessions, a sampling session and a recognition session. A juvenile mouse (4~8 wks. postnatal) was introduced into the test cage for direct interaction with subject mouse for 5min (sampling phase) and then removed from the test cage. After a 60 min inter session interval, I introduced either the same juvenile mouse or another novel juvenile mouse (recognition phase) into the test cage for 5 min interaction. Sniffing/following and aggressive behaviors were recorded by the ANY-maze software (Muromachi Kikai Co., Ltd., Japan). I

collected 41 NR1-KO littermates for control group, 14 CA1-NR1 KO, 15 CA3-NR1 KO, 8 DG-NR1 KO, 42 CA3-TeTX transgenic mice and 38 transgene negative littermates for social memory test.

For the DREADDs cohorts, all mice received either vehicle control (2% v/v DMSO in saline) or clozapine-N-oxide (CNO, 1mg/kg; 4mg/kg for ventral CA3 DREADDs cohort) intraperitoneally 60 min before the sampling session (CNO I) or right after sampling session (CNO II). In the dorsal CA3-CNOI group, I tested SRM in 22 mCherry-injected mice and 21 hM4D(Gi)-injected mice. In dorsal CA3-CNOII group, I tested 28 mCherry-injected mice and 27 hM4D(Gi)-injected mice. In both ventral CA3-CNOI and CNOII group, I tested 6 mCherry-injected mice and 6 hM4D(Gi)-injected mice. The sniffing time in the sampling session and recognition session were compared using paired *t* test, which are grouped by the genetic background or viral expression. Social recognition memory (SRM) index was calculate for the evaluation of memory integrity. The calculation refers to the following formula:

$$\frac{time(recognition\ phase)-time(sampling\ phase)}{time\ (recognition\ phase)+time(sampling\ phase)} \quad \text{(Formula 1)}$$

I was blind to the genotype or virus group during the behavior test.

Object Recognition Memory Test

I refer to Leger's without-habituation protocol[61] and evaluate the performance by calculating the object recognition memory index (ORM index). In brief, the without-habituation protocol has two phases, the sampling phase and the recognition phase. Before the test, all subject mice were allowed to habituate the test room for 30 min. In the sampling phase, subject mice were freely exploring two identical objects (so called Object A) for 5 min and the total sampling time toward two objects was recorded. After 1 hour interval, subject mice start the 5 min recognition

phase, in which one of the object was replaced by a novel object (so called Object B), the exploration time to each object was recorded. Before calculating the ORM index, I first evaluated the total sampling time, and if the sampling time is shorter than 10sec, I exclude the animal for further analysis. The ORM index calculation follows the following formula:

$$\frac{time(object\ B)-time(object\ A)}{time(object\ B)+time(object\ A)} \quad \text{(Formula 2)}$$

I then compared the ORM index of each group of subject mice.

Social Choice Test

Mice were habituated to the test environment for 30min prior to the test. The social choice test is comprised of two 5-min sessions: a sociability session and a social novelty session with an inter session interval of 60min. For the sociability session, one juvenile mouse (younger than 8 wks. postnatal) and one neutral object were firstly introduced into the wired cage of the test cage. Then I introduced subject mice into the test cage for 5min of free exploration. The time of investigation of each cage was recorded. After completing the sociability session, subject mice and juvenile mouse were returned to their home cage. After 60-min, I introduced two juvenile mice into the wired cage inside the test cage, one was the same juvenile mouse from the sociability session and the other was a novel juvenile mouse. Subject mice were then introduced into the test cage for 5-min freely exploration; same as in the sociability session, the investigation time to each wired cage was recorded. I was blind to the genetic background or virus injection of all mice to avoid evaluation bias.

Immunohistochemistry and Microscopy

Mice were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M sodium phosphate buffer. Brains were fixed in 4% PFA overnight and 50μm thick coronal vibratome sections or

cryostat sections were prepared.

To verify the NR1 gene deletion in CA1-NR1 KO, CA3-NR1 KO and DG-NR1 KO mice, I conducted in situ hybridization using NR1 probe (FANTOM clone 4732448C07) labeled with fluorescein according to previously described protocol[21, 60]. Fluorescence images (5X Objective) were collected on Leica DM6000B epifluorescence microscope.

For DREADDs expression evaluation, brain sections were selected along the rostral-caudal axis. Fluorescence images were acquired on Olympus Fluoview FV10i confocal microscope, using multi-area time-lapse function. Multi-area time-lapse images were stitched with Olympus Fluoview ASW software.

Statistical Analysis

As briefly mentioned above, two-tail paired student *t* test was applied to compare the exploration time in the sampling session and the recognition session within the same genetic background. For SRM index comparison across genotypes, ordinary one-way ANOVA test was applied. Mixed ANOVA test (repeated 2-way ANOVA) was applied to compare the social recognition difference across viral injection groups. Uncorrected Fisher's LSD was applied for multiple comparison after ANOVA test. I also provide the effect size (Cohen's d) and its 95% confidence interval for each group of SRM index to demonstrate the effect of each manipulation to the social recognition memory. The effect conventions are: d = 0.2 indicates small effect, d = 0.5 indicates medium effect and d = 0.8 implies large effect[62]. Noted that if the sample size were different during comparison, such as the comparing control SRM index to CA3-NR1 KO SRM index, I then calculated the Hedges' g instead of the Cohen's d to correct sample size bias of Cohen's d. Data plotting and analysis were performed with GraphPad Prism Ver.5 (GraphPad

Software, USA) and Python numpy, scipy stats packages. In addition to the *t* test for classical null hypothesis significance testing (NHST), it's also been suggested to use Bayesian estimation for a better inference[63]; therefore, I implemented Bayesian estimation into our data analysis by using PyMC3 package in Python[64]. The mean effect size and 95% highest probability distribution (HPD) were presented for each comparison.

Chapter 3 Results

Social memory was impaired in CA1 and CA3 NR1-KO mice

The *NR1* gene is essential for NMDA-dependent LTP and is required for spatial memory. However, the involvement of synaptic plasticity for non-spatial memory, especially for social recognition memory, has not been fully addressed. To test whether the integrity of social recognition memory requires hippocampal subregion plasticity, I used region specific NR1-KO mice to survey this question. The social recognition memory test paradigm (Fig. 8) was applied. In brief, after 30 min test room habituation, subject mice were allowed to interact with a novel

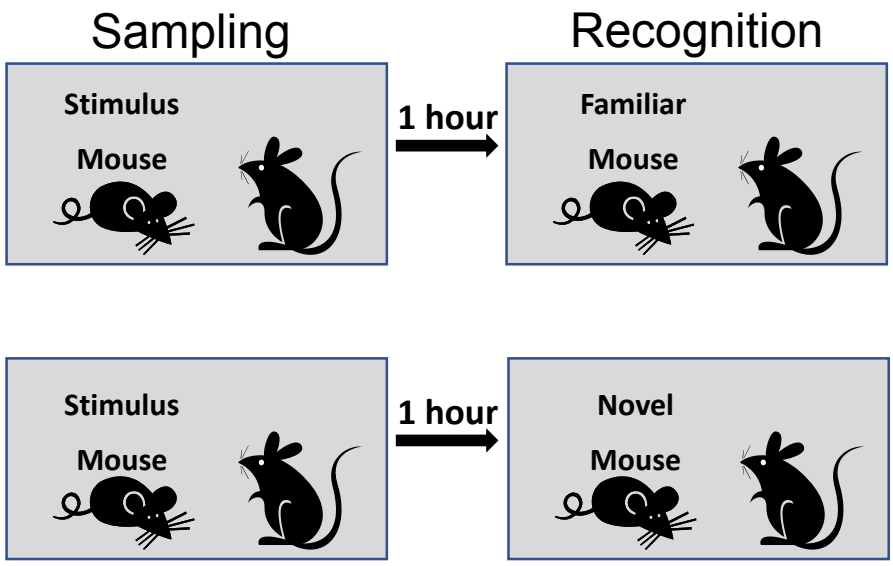


Figure 8. SRM test paradigm. Direct interaction

juvenile mouse for 5 min (sampling session; Sti., indicating the interaction to the stimulus mouse), followed by a 60min inter session interval and then allowed to interact with either the same juvenile mouse in the recognition session (Fam., indicating same, familiar mouse) or with another novel juvenile mouse (Nov., indicating second novel mouse) for 5min. Time spent on social interaction (sniffing and following) was recorded as the index for social memory. For the mouse, the innate behavior in this test is to spend less time on social investigation with a

familiar opponent; therefore, the degree of time difference between two sessions is referred to social memory, which is first introduced by Thor and Holloway[5]. Across all the hippocampal NR1-KO subjects, I found that both CA1 and CA3 NR1-KO mice did not reduce social investigation toward familiar juvenile opponents in the second session, but the identical manipulation in the DG NR1-KO subjects showed profound reduction of social investigation toward familiar opponent (**Fig. 9**). The exploration time between two sessions for each experimental group were tested by paired *t-test*. Control group (Sti: 37.53 ± 3.006; Fam.: 24.76 ± 3.028, paired *t* test, $p < 0.001$; n = 41; **Fig. 9A**) and DG-NR1 KO mice (Sti: 42.43 ± 8.718; Fam.: 19.50 ± 4.827, paired *t* test, $p < 0.003$; n = 8; **Fig. 9D**) showed significant reduction of investigation in the recognition session. However, the exploration time of CA1-NR1 KO mice (Sti: 44.21 ± 7.529; Fam.: 35.99 ± 8.967, paired *t* test, $p = 0.220$; n = 14; **Fig. 9B**) and CA3-NR1 KO mice (Sti: 32.36 ± 6.002 Fam.: 30.48 ± 6.243 paired *t* test, $p = 0.696$; n = 15; **Fig. 9C**) did not show statistically reduction in the recognition session. Comparing the SRM index, a normalized measure of habituation (**Formula 1**), across genetic backgrounds revealed a significant effect of genetic background and clear impairment in the CA3-NR1 KO mice compare to controls (Ctrl: -0.228 ± 0.04, CA1-NR1 KO: -0.104 ± 0.07, CA3-NR1 KO: -0.065 ± 0.07, DG-NR1 KO: -0.306 ± 0.05. One-way ANOVA $F(3, 74)= 3.883$, $P = 0.012$. Multiple comparison with uncorrected Fisher's LSD: Ctrl vs. CA1-NR1 KO: $p = 0.108$; Ctrl vs. CA3-NR1 KO: $p = 0.031$; Ctrl vs. DG-NR1 KO: $p = 0.100$; **Fig.9E**). In a separated experiment social investigation toward a novel opponent was also recorded (**Fig. 8**). To demonstrate the magnitude of difference between mice from each genetic background, I further computed the 95% CI of the effect size (Hedge's *g*) for the SRM index of each experimental group comparing

to controls (CA1-NR1 KO: -0.49 ± 0.61, CA3-NR1 KO: -0.655 ± 0.60, DG-NR1 KO: 0.683 ± 0.77; **Fig 10E**). Interestingly, this behavioral deficit is specific to familiar recognition. None of the KO animal showed behavioral impairment to novel social opponent recognition (**Fig. 11**). The statistical parameters of SRM indices of each genotype were summarized in table 1 and table 2 for familiar and novel recognition respectively. It is worth mentioning that only the 95% CI of CA3-NR1 KO mice familiar SRM index effect size excluded zero effect. Both the post *hoc* multiple comparison and effect size captured the familiar recognition memory deficit in CA3-NR1 KO mice. To ask if these impairments we observed from the CA3-NR1 KO mice were specific to social stimulus or were reflective general recognition deficit we subjected these animals to further recognition testing.

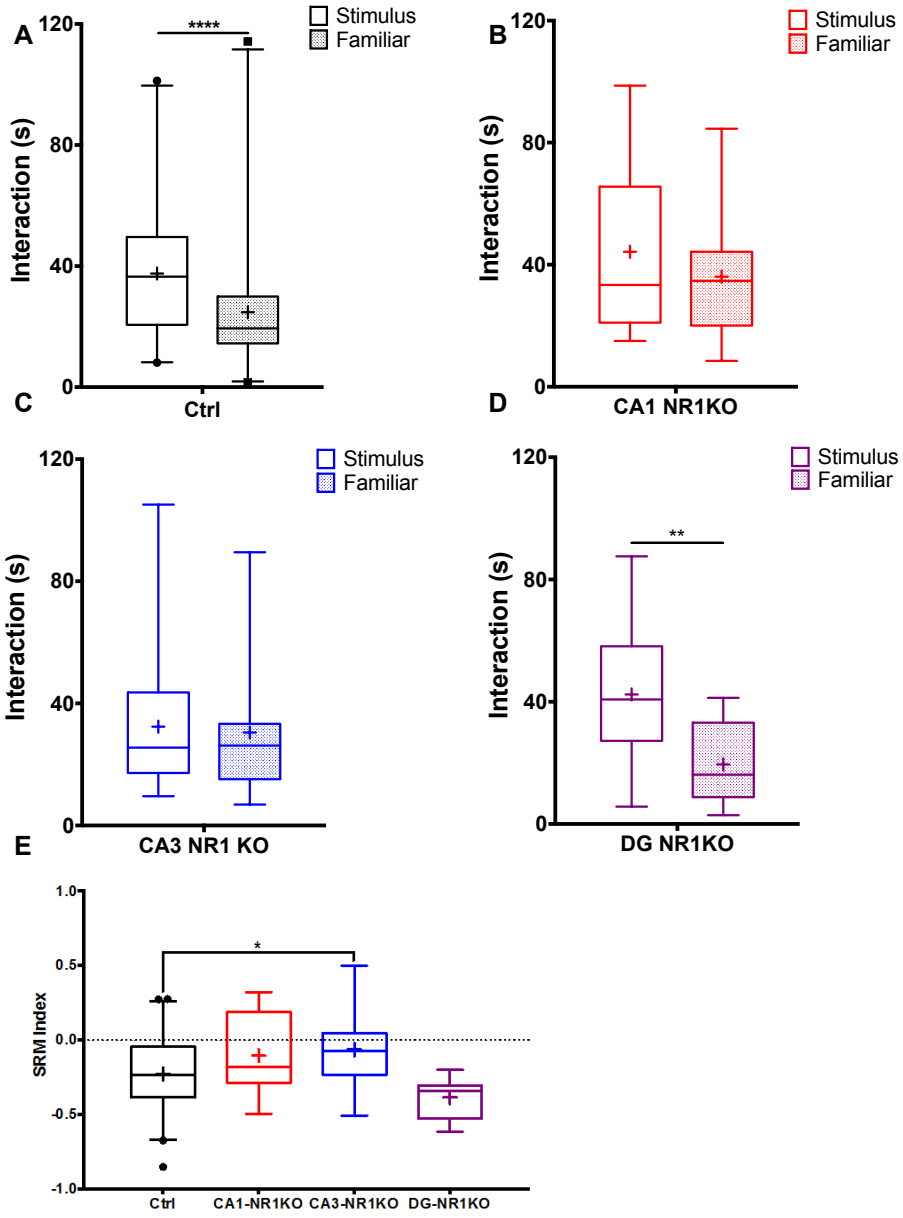


Figure 9. Hippocampal CA1 or CA3 NR1 knockout impairs social recognition of familiar opponent. Interaction time toward the same social opponent of (A) control littermate (Sti: 37.53 ± 3.006; Fam.: 24.76 ± 3.028, paired *t* test, $p < 0.001$; n = 41) (B) CA1-NR1 KO (Sti: 44.21 ± 7.529; Fam.: 35.99 ± 8.967, paired *t* test, $p = 0.220$; n = 14), (C) CA3-NR1-KO (Sti: 32.36 ± 6.002 Fam.: 30.48 ± 6.243 paired *t* test, $p = 0.696$; n = 15) and (D) DG-NR1 KO mice (Sti: 42.43 ± 8.718; Fam.: 19.50 ± 4.827, paired *t* test, $p < 0.003$; n = 8). (E) SRM index of different KO animals. (Ordinary one-way ANOVA: $F(3, 74) = 3.883$; $p = 0.012$. Multiple comparison with uncorrected Fisher's LSD: Ctrl vs. CA1-NR1 KO: $p = 0.108$; Ctrl vs. CA3-NR1 KO: $p = 0.031$; Ctrl vs. DG-NR1 KO: $p = 0.100$).

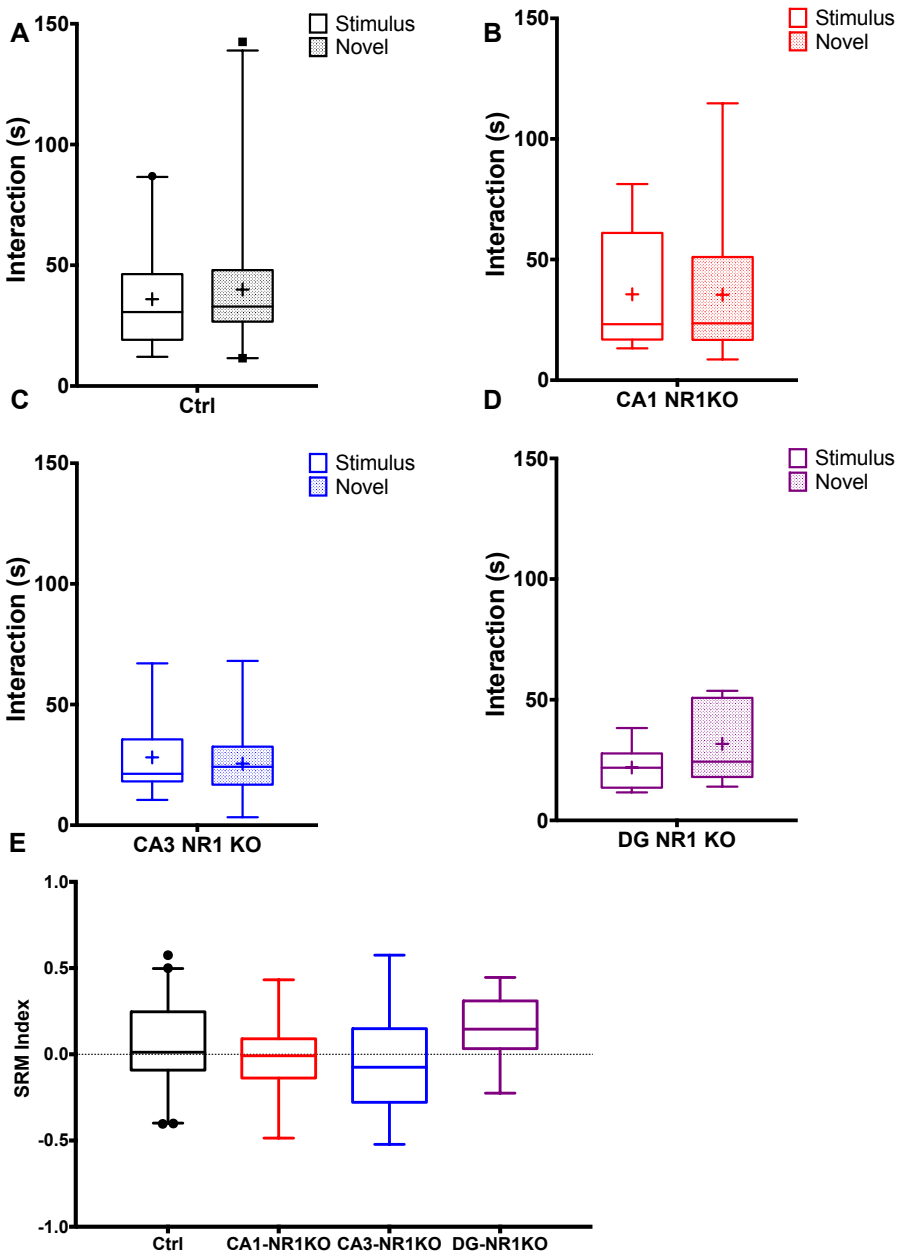


Figure 10. Hippocampus subregion NR1 knockout of does not affect social recognition of novel opponent. Interaction time toward the same social opponent of (A) control littermate ($p = 0.211$, paired t test) (B) CA1-NR1 KO ($p = 0.963$, paired t test), (C) CA3-NR1-KO ($p = 0.635$, paired t test) and (D) DG-NR1 KO mice ($p = 0.079$, paired t test). (E) SRM index of different KO animals (Ordinary one-way ANOVA: $F(3, 74) = 1.65$; $p = 0.185$).

Table 1. Summary of familiar SRM index statistics of NR1 KO animals.

Familiar SRM Index	mean	s.e.m.	n	95% CI of Effect size
Ctrl	-0.228	0.039	41	(Hedge's g)
CA1 NR1K-KO	-0.104	0.074	14	-0.490 ± 0.614
CA3 NR1-KO	-0.065	0.068	15	*-0.655 ± 0.604
DG NR1-KO	-0.386	0.049	8	0.683 ± 0.770

The 95% CI is estimated and represented in effect size ± 1.96*sed. * indicated the 95% CI exclude 0 effect.

Table 2. Summary of novelty SRM index statistics of NR1 KO animals.

Novelty SRM Index	mean	s.e.m.	n	95% CI of Effect size
Ctrl	0.050	0.039	41	(Hedge's g)
CA1 NR1K-KO	-0.025	0.063	14	0.302 ± 0.609
CA3 NR1-KO	-0.069	0.076	15	0.450 ± 0.598
DG NR1-KO	0.151	0.074	8	-0.412 ± 0.762

The 95% CI is estimated and represented in effect size ± 1.96*sed. All the 95% CI include 0 effect.

Recognition memory impairment in CA3-NR1 KO mice is specific for social opponent.

To test whether the recognition deficit in CA3-NR1 KO mice is general impairment in recognition or is specific toward social stimuli, I applied the well-established object recognition protocol to test CA3-NR1-KO mice behavioral performance (**Fig. 11A**). In brief, subject mice were allowed to freely explore two identical objects for 5 min during the sampling session. After 1hr interval, I substituted one object to a novel object at the same position and recorded

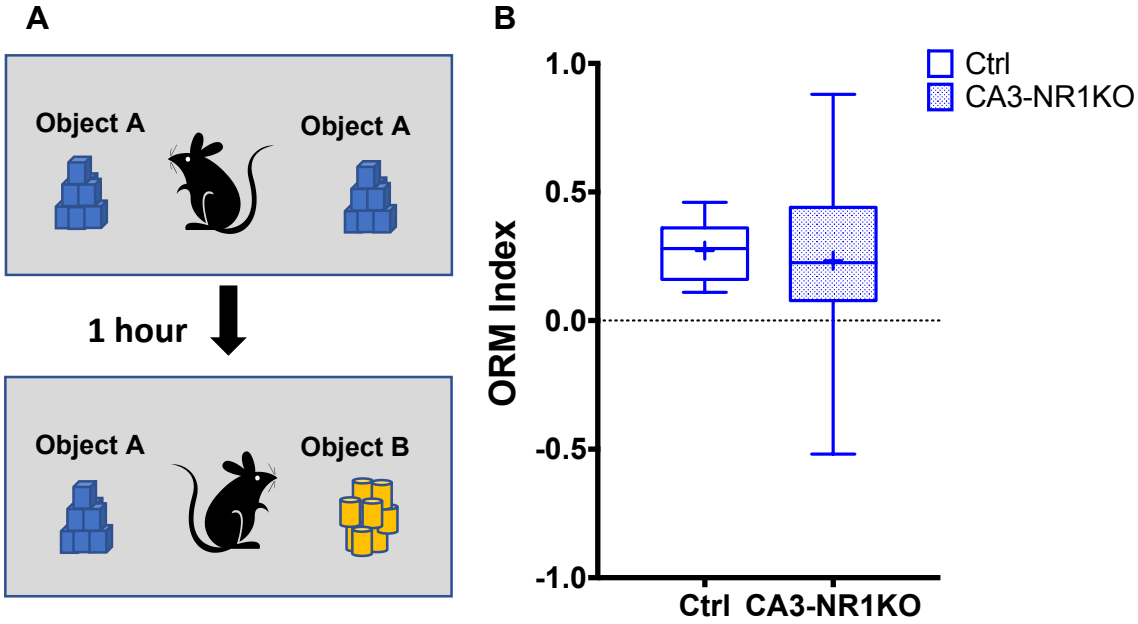


Figure 11. Object recognition memory is intact in CA3-NR1 knockout mice. (A) The object recognition memory test paradigm. (B) ORM index of CA3-NR1 KO mice (Ctrl: 0.2718 ± 0.0328; CA3-NR1 KO: 0.2325 ± 0.1405, unpaired *t* test, $p = 0.756$; Ctrl, n = 11; CA3-NR1 KO, n = 8).

the exploration time. The ORM index was then calculated (**Formula 2**), and the results suggested that the object recognition performance of CA3-NR1 KO mice was indistinguishable from the littermate control (Ctrl: 0.2718 ± 0.0328; CA3-NR1 KO: 0.2325 ± 0.1405, unpaired *t* test, $p = 0.756$; Ctrl, n = 11; CA3-NR1 KO, n = 8; **Fig. 11B**). Table 3 summarized the ORM

indices' statistics, where the 95% CI of effect size did not exclude the zero-effect which indicating there is no behavioral difference between groups. The other concern was the NR1 KO animals have lower motivation in interacting with social opponents, therefore, to confirm whether these mutants had underlying changes of baseline interest in social interaction I applied a well-established social choice task (**Fig 12A**). In brief, subject mice are given the choice to

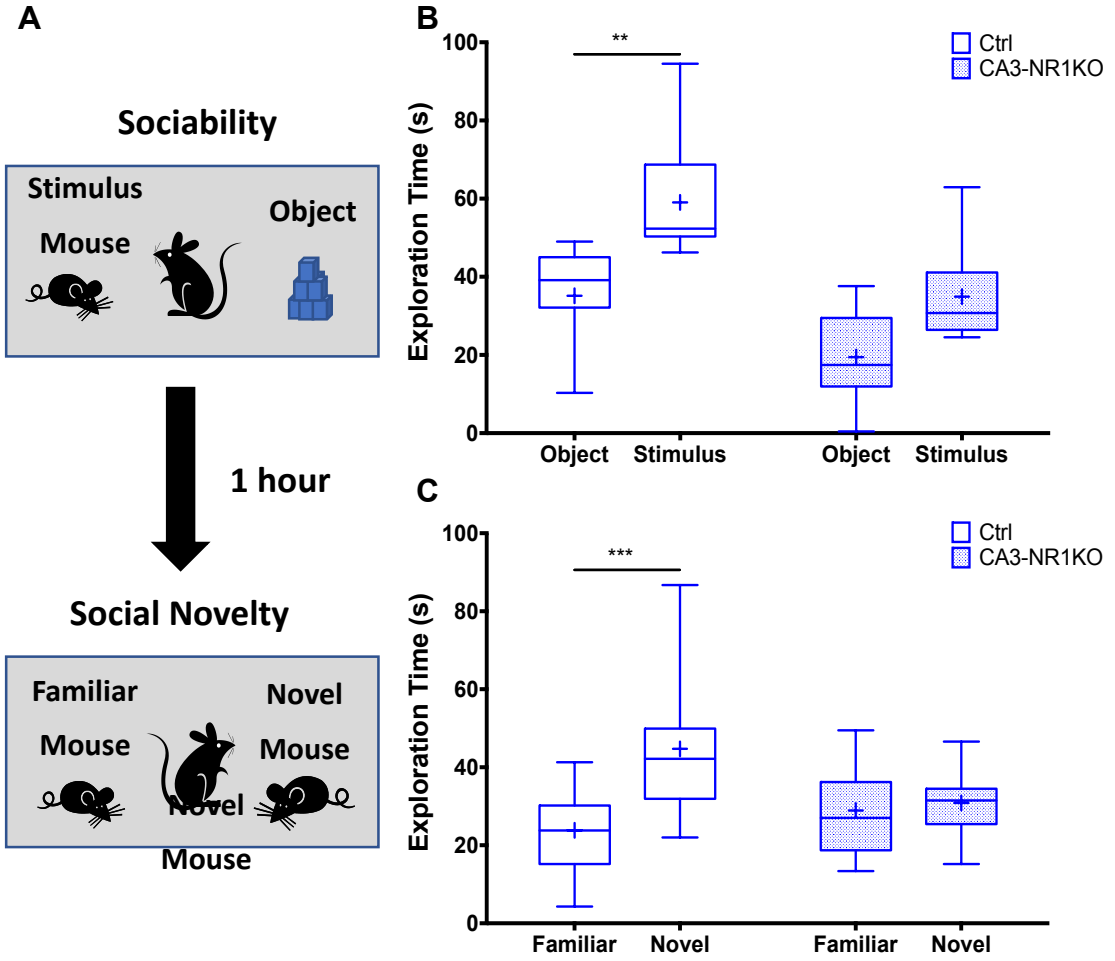


Figure 12. CA3-NR1 KO mice show social novelty impairment. (A) Social choice test paradigm. (B) Sociability session. Two-way RM ANOVA: Genotype × Social stimulus $F(1, 17) = 0.7733$, $p = 0.391$; Genotype: $F(1, 17)$: 26.98, $p < 0.001$; Social stimulus: $F(1, 17) = 16.95$, $p < 0.001$ Multiple comparison of Object vs. Social stimulus with uncorrected Fisher's LSD among genotypes: Ctrl: $p = 0.001$; CA3-NR1 KO: $p = 0.048$. (C) Social novelty session. (Two-way RM ANOVA: Genotype × Social stimulus $F(1, 17) = 12.09$, $p = 0.003$; Genotype: $F(1, 17)$: 0.6973, $p = 0.415$; Social stimulus: $F(1, 17) = 17.58$, $p < 0.001$). Ctrl, n = 11; CA3-NR1 KO, n = 8

explore a novel conspecific or a novel object in the sociability session and to explore another novel conspecific or the familiar one in the social novelty session. The sociability session and social novelty session are separated by 1hr interval, same as in the previous behavioral tasks. The behavioral performance of CA3-NR1 KO animals were indistinguishable from the control littermates in the sociability session, showing a greater preference in exploring the novel conspecific than exploring the novel object. The behavioral performance among genotypes were analyzed by repeated measure two-way ANOVA (Genotype × Social stimulus $F(1, 17) = 0.7733$, $p = 0.391$; Genotype: $F(1, 17)$: 26.98, $p < 0.001$; Social stimulus: $F(1, 17) = 16.95$, $p < 0.001$). Multiple comparisons of Object vs. Stimulus with uncorrected Fisher's LSD among genotypes: Ctrl: $p = 0.001$; CA3-NR1 KO: $p = 0.048$; **Fig. 12B**). These results suggested that CA3-NR1 KO animals had comparable level of interests in exploring social opponent with control animals. However, in the social novelty session, the CA3-NR1 KO animals failed to show the preference toward the novel mouse while the controls showed significantly stronger preference to novel mouse than familiar mouse (Two-way RM ANOVA: Genotype × Social stimulus $F(1, 17) = 12.09$, $p = 0.003$; Genotype: $F(1, 17)$: 0.6973, $p = 0.415$; Social stimulus: $F(1, 17) = 17.58$, $p < 0.001$; **Fig. 12C**), which suggested animals without CA3 plasticity might not able to distinguish the familiar conspecific from novel conspecific. Table 4 and table 5 summarized the statistical measurements of sociability indices and social novelty indices, where the 95% CI of effect size of social novelty indices exclude the zero-effect, which indicates the behavioral performance in CA3-NR1 KO mice is different from controls.

In summary, my results indicated that loss of CA3 plasticity specifically impaired social recognition memory but has no effect on the object recognition memory. The social recognition

impairment in CA3 plasticity deficiency animals might be because of encoding impairment or retrieval failure; however, the genetic manipulation approach has no way to dissecting the CA3 dysfunction in encoding or in retrieval of social recognition memory. Therefore, an activity manipulation with better temporal control is needed to address whether the CA3 activity involves in encoding or in retrieval in terms of social recognition memory.

Table 3. Summary of ORM index statistics.

ORM Index	mean	s.e.m.	n	95% CI of Effect size
Ctrl	0.2718	0.033	11	(Hedge's g)
CA3 NR1-KO	0.2325	0.140	8	0.152 ± 0.465

The 95% CI is estimated and represented in effect size ± 1.96*sed.

Table 4. Summary of sociability index statistics.

Sociability Index	mean	s.e.m.	n	95% CI of Effect size
Ctrl	0.261	0.070	11	(Hedge's g)
CA3 NR1-KO	0.336	0.119	8	-0.253 ± 0.467

The 95% CI is estimated and represented in effect size ± 1.96*sed.

Table 5. Summary of social novelty index.

Social novelty Index	mean	s.e.m.	n	95% CI of Effect size
Ctrl	0.313	0.060	11	(Hedge's g)
CA3 NR1-KO	0.049	0.041	8	*1.48 ± 0.530

The 95% CI is estimated and represented in effect size ± 1.96*sed. * indicated the 95% CI exclude 0 effect.

AAV infection can be specifically targeted across dorsal-ventral CA3

To acutely manipulate CA3 activity, I used a chemogenetic approach expressing the hM4D(Gi) DREADD system. Before the CA3 specific activity control, a pilot

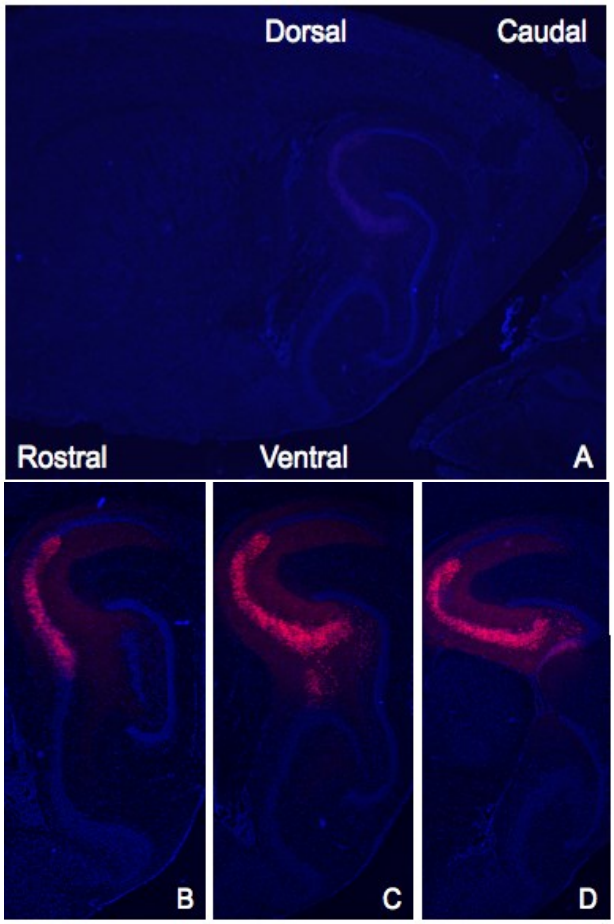


Figure. 13. Genetic targeting of CA3-Cre mouse. All panels showed DAPI counterstain (Blue) of sagittal section of CA3 Cre-transgenic mice injected with AAV.DIO.mCherry (Red) into dorsal CA3. (A) 1.8X magnification. (B to D) Sagittal sections selected from lateral to medial. DAPI image merged with mCherry signal, 5X magnification.

experiment for expression specificity was tested. I combined CA3-specific Cre enzyme transgenic animals with DIO.mCherry AAV injection into dorsal CA3. Two weeks post-viral injection, I confirmed the mCherry expression by cutting the mouse brain in the sagittal plane and observing mCherry expression. In the brain sections, mCherry was exclusively expressed in dorsal CA3 based on our injection coordinates (**Fig. 13B-D**).

Therefore, the combination of CA3-Cre transgenic mice and cre-dependent hM4D(Gi) expressing viruses help me to address the question which CA3 activity involves in social memory encoding or social memory retrieval.

Social memory deficit was found in CA3 inactivated mice

As I mentioned above, the chronic deletion of CA3 NMDA-dependent plasticity revealed a clear SRM deficit, however this approach does not allow me to distinguish contributions to

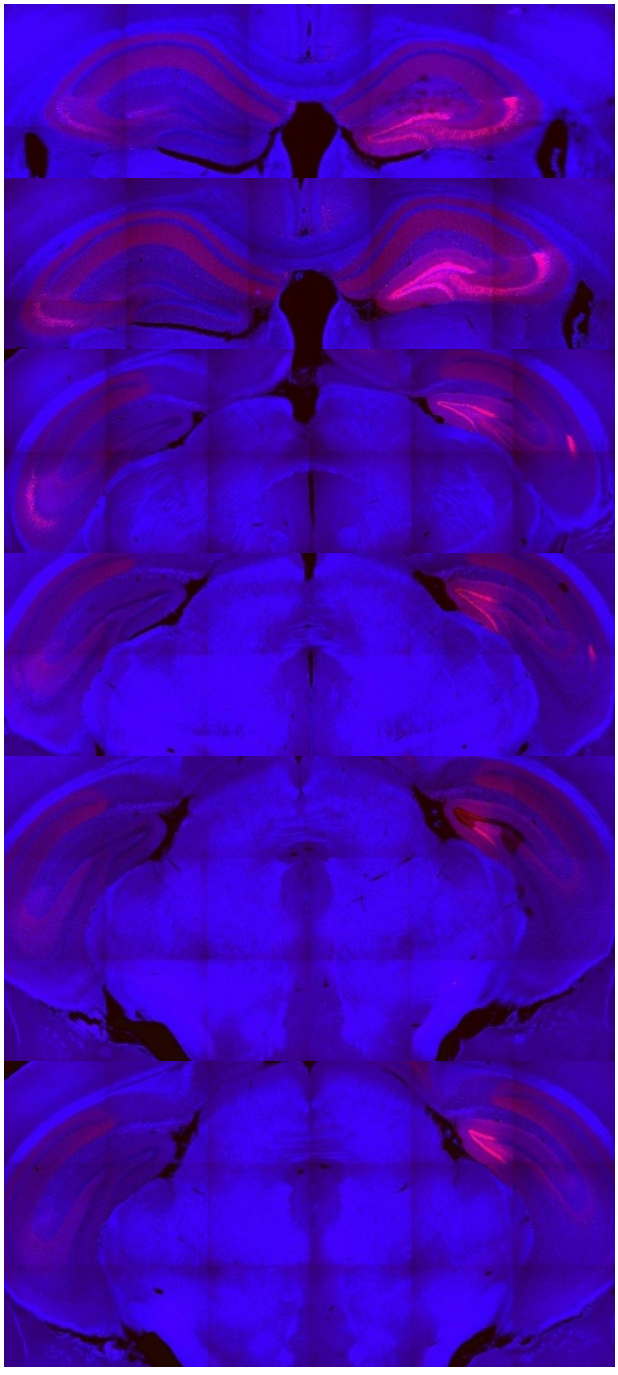


Figure 14. Expression of DIO.hM4D(Gi)-mCherry in dorsal hippocampus. Hippocampal coronal sections were selected across rostral to caudal direction. Images were taken by 10X lens.

encoding versus expression of the memory. Thus, I injected the inhibitory DREADD (hM4D(Gi)) into CA3-Cre transgenic mouse brain and test their SRM performance. The hM4d(Gi) receptors are exclusively activated by clozapine-N-oxide (CNO), which caused neuronal hyperpolarization. By using this approach, I can address which phases of the task require CA3 activity by alternating the CNO introduction timing during SRM test. In the histological examination, hM4d(Gi)-mCherry expression was mainly found in dorsal hippocampus CA3 and DG (**Fig. 14**) similar to previous description[51]. Two different CNO protocols were used in the SRM test paradigm (**Fig. 15**). In the CNO I group the subjects

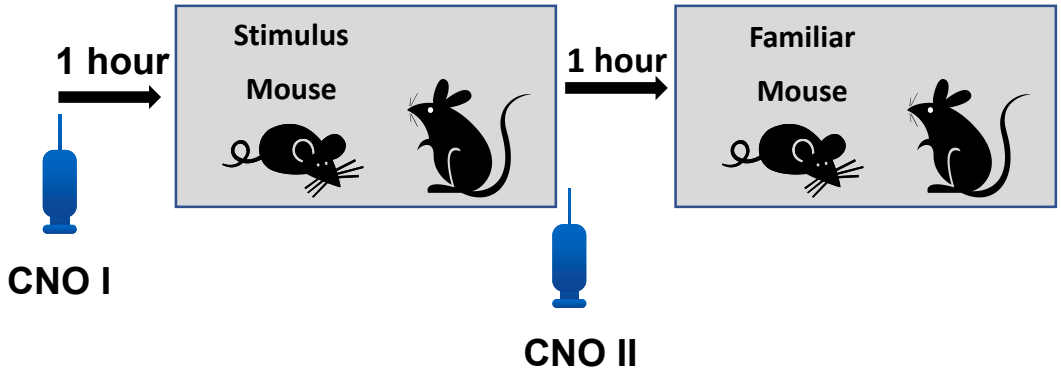


Figure 15. SRM test paradigm for CA3-DREADDs animal. Animals were received CNO injection either 1hr before the SRM test (CNO I) or right after the sampling phase (CNO II).

received CNO injection 60min before the sampling session; where in the CNO II group the subjects received CNO injection right after the recognition session. The rationale of these injection timings is determined by the temporal scale of neural activity shutdown. From our previous findings, neurons were inactivated after 15min to 20min CNO injection and the effect can last few hours[24]. Therefore, the CA3 activity will putatively shut down from the encoding phase to the following retrieval phase in the CNO I group and will shut down during the retrieval in the CNO II group. However, neither my results from the CNO I group (Two-way

RM ANOVA: Virus type × Social stimulus $F(1, 40) = 0.1168$, $p = 0.734$; Virus type: $F(1, 40)$: 1.271, $p = 0.266$; Social stimulus: $F(1, 40) = 36.42$, $p < 0.001$. Multiple comparisons of stimulus vs. familiar with Fisher's LSD among viral types: mCherry + CNO: $p < 0.001$; hM4d(Gi) + CNO: $p < 0.001$; **Fig. 16A**) nor the CNO II group (Two-way RM ANOVA: Virus type × Social stimulus $F(1, 52) = 0.003329$, $p = 0.954$; Viral type: $F(1, 52)$: 7.827, $p = 0.007$; Social stimulus: $F(1, 52) = 39.36$, $p < 0.001$. Multiple comparisons of stimulus vs. familiar with Fisher's LSD: mCherry + CNO: $p < 0.001$; hM4d(Gi) + CNO: $p < 0.001$; **Fig. 16C**) showed SRM impairment. The *t* test comparisons of SRM index among viral types showed no difference both in CNO I (mCherry + CNO: -0.294 ± 0.05; hM4d(Gi) + CNO: -0.180 ± 0.06; unpaired *t* test: $p = 0.189$. CNO I: nCherry, n = 22; hM4d(Gi), n = 21; **Fig. 16B, Table 6**) and CNO II (mCherry + CNO: -0.256 ± 0.04; hM4d(Gi) + CNO: -0.207 ± 0.05; unpaired *t* test: $p = 0.439$. CNO I: nCherry, n = 28; hM4d(Gi), n = 27; **Fig. 16D, Table 7**) group.

Table 6. Summary of dCA3 CNOI SRM index statistics.

SRM Index	mean	s.e.m.	n	95% CI of Effect size
mCherry + CNOI	-0.294	0.053	22	(Hedge's g)
hM4D(Gi) + CNOI	-0.180	0.064	21	* -0.409 ± 0.308

The 95% CI is estimated and represented in effect size ± 1.96*sed. * indicated the 95% CI exclude 0 effect.

Table 7. Summary of dCA3 CNOII SRM index statistics.

SRM Index	mean	s.e.m.	n	95% CI of Effect size
mCherry + CNOII	-0.256	0.042	28	(Hedge's g)
hM4D(Gi) + CNOII	-0.207	0.045	27	-0.210 ± 0.270

The 95% CI is estimated and represented in effect size ± 1.96*sed.

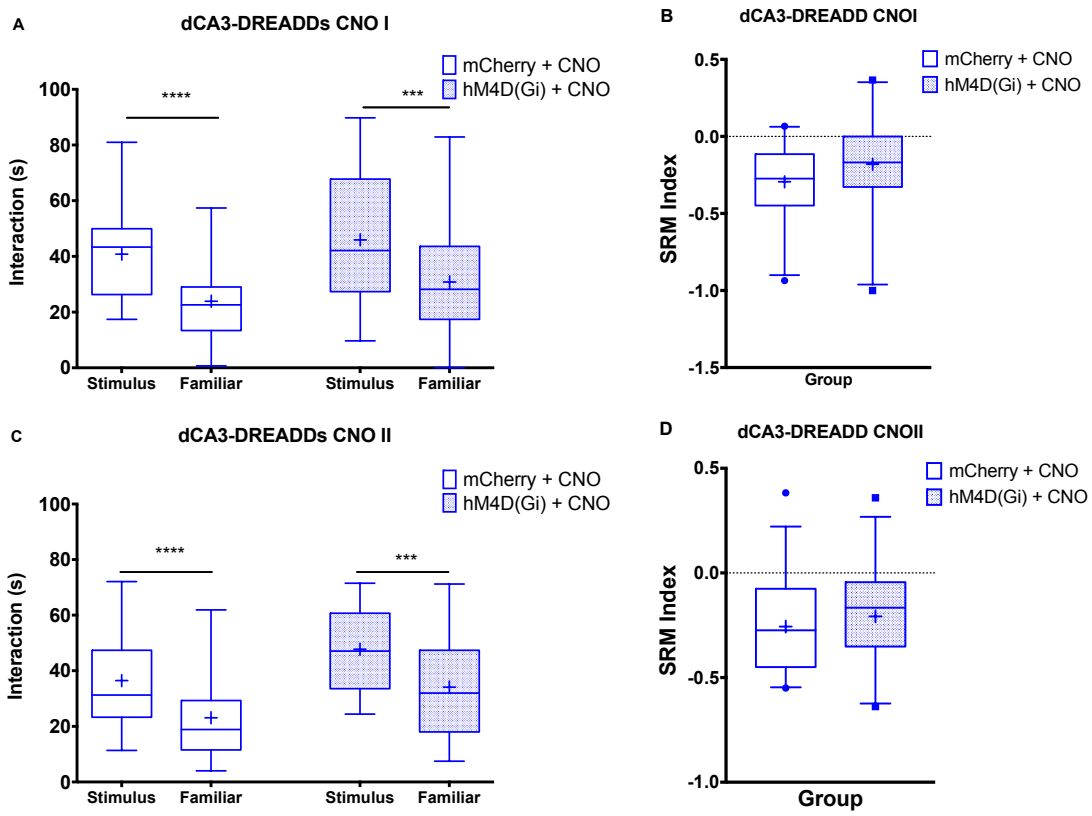


Figure 16. Activity manipulation on dorsal CA3 does not impair mouse social recognition memory. (A) Interaction time from CNO I session (Two-way RM ANOVA: Viral type × Social stimulus $F(1, 40) = 0.1168$, $p = 0.734$; Viral type: $F(1, 40)$: 1.271, $p = 0.226$; Social stimulus: $F(1, 40) = 36.42$, $p < 0.001$; Multiple comparisons of stimulus vs. familiar with Fisher's LSD among viral types: mCherry + CNO: $p < 0.001$; hM4d(Gi) + CNO: $p < 0.001$). (B) SRM index of CNO I session (mCherry + CNO: -0.294 ± 0.05; hM4d(Gi) + CNO: -0.180 ± 0.06; unpaired *t* test: $p = 0.189$; mCherry + CNO, n = 22, hM4d(Gi) + CNO, n = 21). (C) Interaction time from CNO II session. (Two-way RM ANOVA: Virus type × Social stimulus $F(1, 52) = 0.003329$, $p = 0.954$; Viral type: $F(1, 52)$: 7.827, $p = 0.007$; Social stimulus: $F(1, 52) = 39.36$, $p < 0.001$. Multiple comparisons of stimulus vs. familiar with Fisher's LSD: mCherry + CNO: $p < 0.001$; hM4d(Gi) + CNO: $p < 0.001$). (D) SRM index of CNO II session (mCherry + CNO: -0.256 ± 0.04; hM4d(Gi) + CNO: -0.207 ± 0.05; unpaired *t* test: p = 0.439. CNO I: nCherry, n = 28; hM4d(Gi), n = 27).

I speculated that the different behavioral outcome between CA3-NR1KO mice and DREADD mice might be because of the functional heterogeneity across the CA3 dorsal-ventral axis. Since

we injected hM4d(Gi)-expressing AAV into the dCA3, our manipulation of CA3 activity was limited to dCA3. However, the CA3-NR1KO was complete across the dorsal-ventral axis. Based on this speculation, I hypothesized that the ventral CA3 might play a more important role in social recognition memory. To test my hypothesis, I injected hM4d(Gi)-expressing AAV into vCA3 (**Fig. 17**). After two weeks for post-surgery recovery, I performed the SRM test followed with the same behavioral paradigm design (**Fig. 15**). The manipulation of vCA3 activity impaired SRM in the CNO I group(Two-way RM ANOVA: Viral type × Social stimulus $F(1, 10) = 3.698.$, $p = 0.083$; Viral type: $F(1, 10)$: 2.57, $p = 0.140$; Social stimulus: $F(1, 10) = 15$, $p = 0.003$; Multiple comparisons of stimulus vs familiar with Fisher's LSD among viral types: mCherry + CNO: $p = 0.002$; hM4d(Gi) + CNO: $p = 0.198$; **Fig. 18A**). The comparison of SRM index also showed significant difference among viral types (mCherry + CNO: -0.370 ± 0.07; hM4d(Gi) + CNO: -0.05 ± 0.05; unpaired *t* test: $p = 0.004$ CNO I: mCherry, n = 6; hM4d(Gi), n = 6; **Fig. 18B**). However, no behavioral impairment was found in CNO II group (Two-way RM ANOVA: Viral type × Social stimulus $F(1, 10) = 0.005358$, $p = 0.9431$; Viral type: $F(1, 10)$: 6.312, $p = 0.0308$; Social stimulus: $F(1, 10) = 19.69$, $p = 0.0013$; Multiple comparisons of stimulus vs. familiar with Fisher's LSD: mCherry + CNO: $p = 0.0097$; hM4d(Gi) + CNO: $p = 0.0115$; **Fig. 18C, Table 8**). Although the SRM index of CNO II group from the hM4d(Gi) animals showed greater variation, there was no significant difference between the mCherry animals and hM4d(Gi) animals (mCherry + CNO: -0.458 ± 0.07; hM4d(Gi) + CNO: -0.286 ± 0.09; unpaired t test: $p = 0.163$ CNO II: mCherry, n = 6; hM4d(Gi), n = 6; **Fig. 18D, Table 9).**

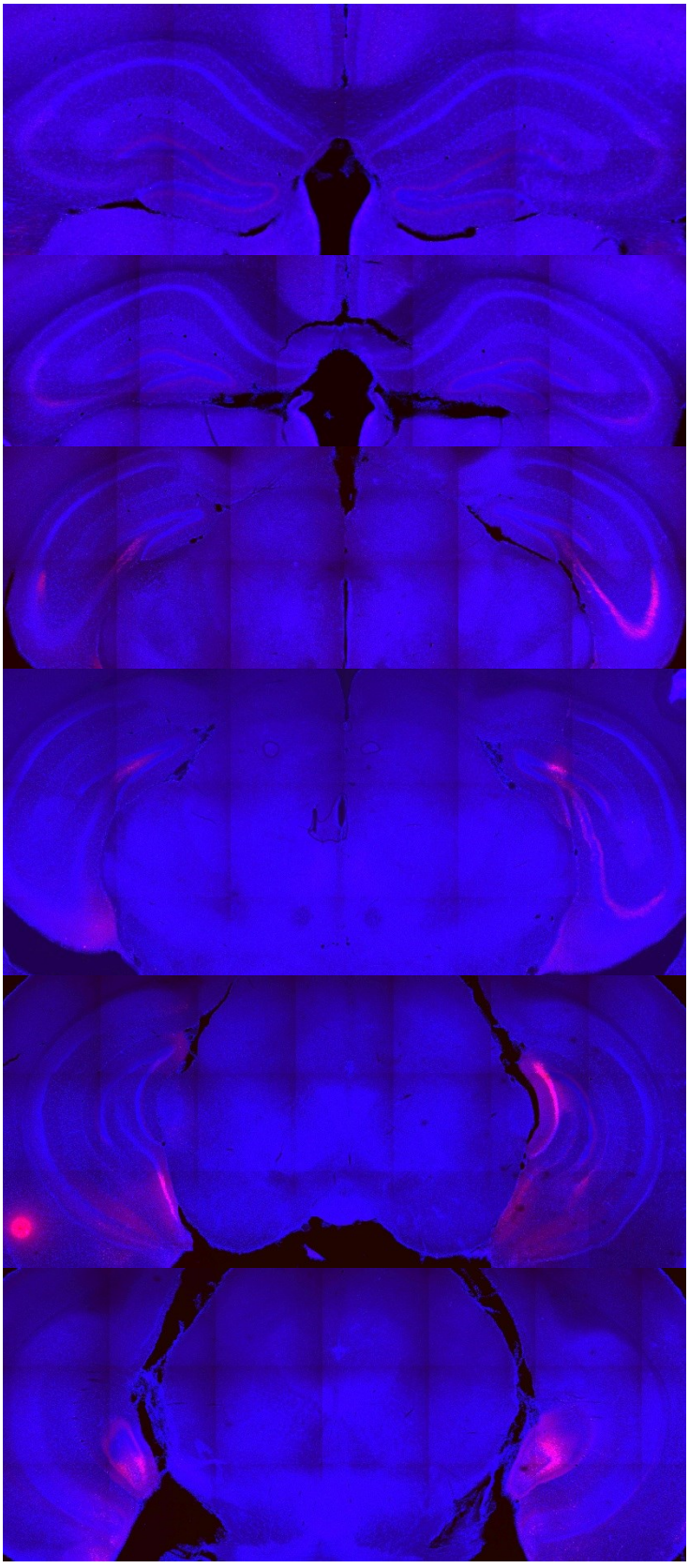


Figure 17. Expression of DIO.hM4D(Gi)-mCherry in ventral hippocampus. Hippocampal coronal sections were selected across rostral to caudal direction. Images were taken by 10X lens.

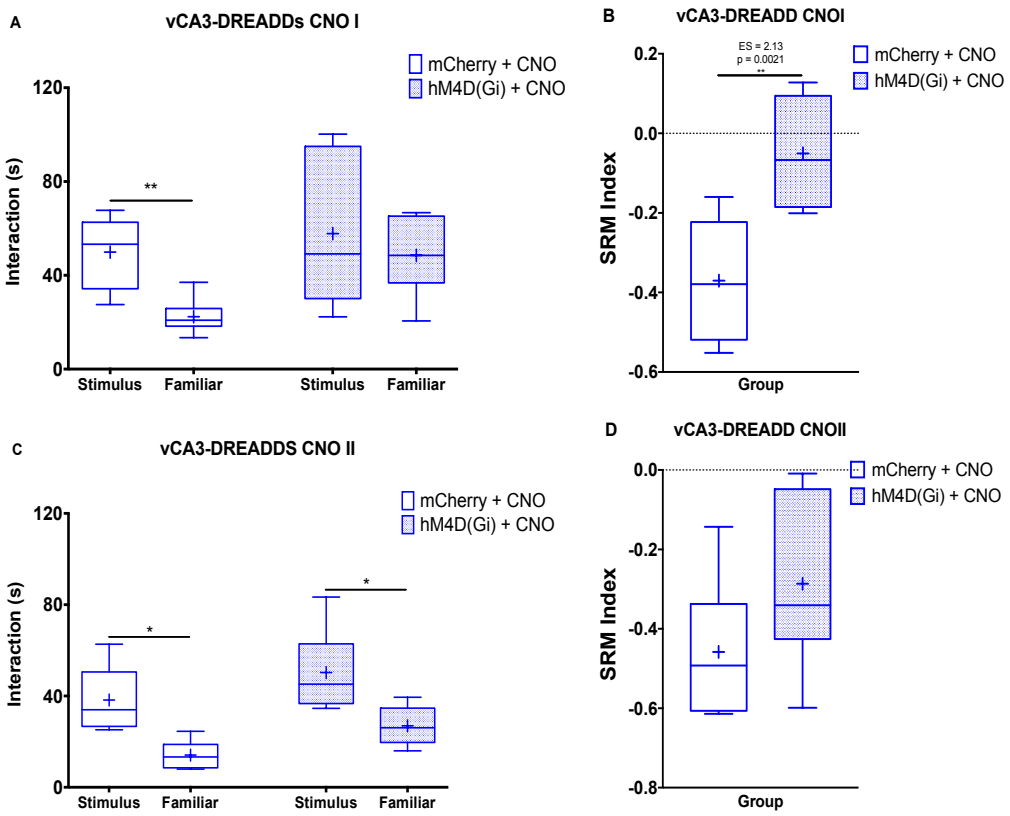


Figure 18. Ventral CA3 inactivation during encoding impairs mouse social recognition memory. (A) Interaction time from CNO I session (Two-way RM ANOVA: Viral type × Social stimulus $F(1, 10) = 3.698.$, $p = 0.083$; Viral type: $F(1, 10)$: 2.57, $p = 0.140$; Social stimulus: $F(1, 10) = 15$, $p = 0.003$; Multiple comparisons of stimulus vs. familiar with Fisher's LSD among viral types: mCherry + CNO: $p = 0.002$; hM4d(Gi) + CNO: $p = 0.198$). (B) SRM index of CNO I session. mCherry + CNO: -0.370 ± 0.07; hM4d(Gi) + CNO: -0.05 ± 0.05; unpaired *t* test: $p = 0.004$. CNO I: mCherry, n = 6; hM4d(Gi), n = 6. (C) Interaction time from CNO II session. (Two-way RM ANOVA: Viral type × Social stimulus $F(1, 10) = 0.005358$, $p = 0.9431$; Viral type: $F(1, 10)$: 6.312, $p = 0.0308$; Social stimulus: $F(1, 10) = 19.69$, $p = 0.0013$; Multiple comparisons of stimulus vs. familiar with Fisher's LSD: mCherry + CNO: $p = 0.0097$; hM4d(Gi) + CNO: $p = 0.0115$). (D) SRM index of CNO II session. mCherry + CNO: -0.458 ± 0.07; hM4d(Gi) + CNO: -0.286 ± 0.09; unpaired *t* test: $p = 0.163$ CNO II: mCherry, n = 6; hM4d(Gi), n = 6.

Together with the results form CA3-NR1 KO animals and DREADD manipulation, I have demonstrated that the chronic loss of CA3 plasticity (CA3-NR1 KO) and acute inactivation of ventral CA3 activity (vCA3-DREADD) led to social recognition memory deficit. Moreover,

the vCA3 activity is critical for encoding phase but dispensable for retrieval. Together with the previous works which demonstrated that the CA2 transmission is required for social memory[12], and vCA1 stores social memory[15], these data support a model in which the ventral hippocampus works together as a functional unit supporting social recognition memory, with CA2 as the modulator. Whether the social information in vCA1 is determined by vCA3 input remains an interesting open question. In addition, considering the theory of CA3 function for memory encoding, the information is encoded in the attractor network, in which the attractor activity is maintained by the CA3 to CA3 transmission. According to these two perspectives, a new hypothesis is then proposed: whether the CA3 transmission is essential for supporting social recognition memory.

Table 8. Summary of vCA3 CNOI SRM index statistics.

SRM Index	mean	s.e.m.	n	95% CI of Effect size
mCherry + CNOI	-0.370	0.061	6	(Cohen's d)
hM4D(Gi) + CNOI	-0.051	0.050	6	* -2.135 ± 0.749

The 95% CI is estimated and represented in effect size ± 1.96*sed. * indicated the 95% CI exclude 0 effect.

Table 9. Summary of vCA3 CNOII SRM index statistics.

SRM Index	mean	s.e.m.	n	95% CI of Effect size
mCherry + CNOII	-0.458	0.065	6	(Cohen's d)
hM4D(Gi) + CNOII	-0.286	0.081	6	-0.869 ± 0.609

The 95% CI is estimated and represented in effect size ± 1.96*sed.

Social memory was not impaired by CA3 transmission blockade

It requires specific blockade of CA3 transmission to test whether it is essential for social memory. To achieve this CA3-specific transmission blockade, I used inducible CA3-TeTX transgenic mice for the SRM behavioral test paradigm. As mentioned in introduction, TeTX transgene expression is induced by DOX withdrawal, hence, I can compare the subjects' SRM performance before and after TeTX expression (**Fig. 19**). In brief, SRM performance of CA3-

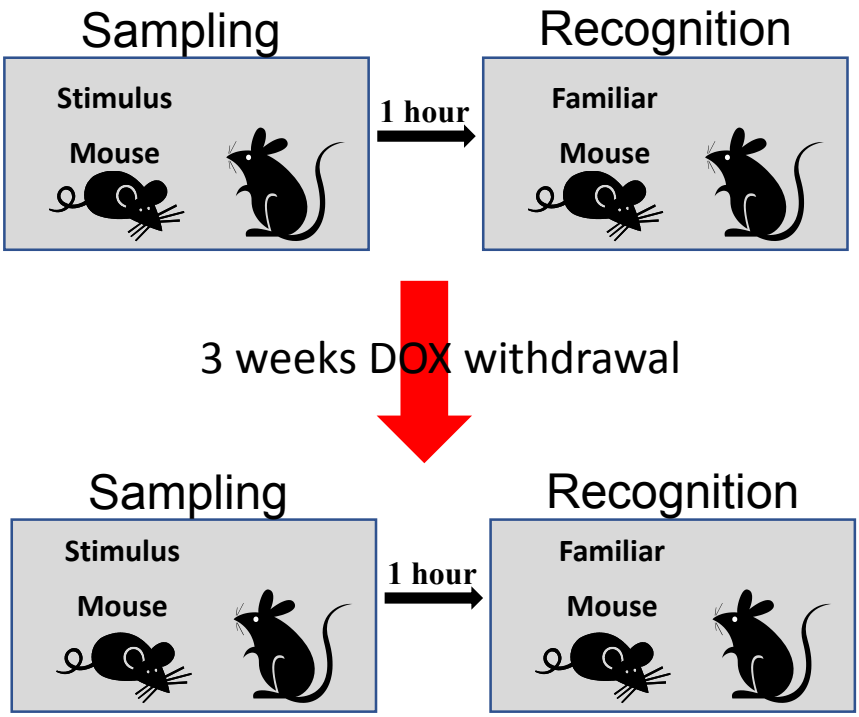


Figure 19. SRM test paradigm-Direct interaction for CA3-TeTX mice.

TeTX mice was first recorded when the subjects were fed 10mg/kg DOX food (On-DOX session). The DOX food was replaced by the regular food for 3weeks. As described previously[32], 3weeks of DOX withdrawal results in a complete loss of CA3 transmission. The CA3-TeTX mice were then again used to test SRM (Off-DOX session). In the On-DOX session, both control mice (Sti: 47.48 ± 4.191; Fam: 33.28 ± 4.315; paired *t* test: $p = 0.022$;n = 31. **Fig. 20A**) and CA3-TeTX mice (Sti: 50.19 ± 4.366; Fam: 32.51 ± 2.668; paired *t* test: $p < 0.001$;

n = 31. **Fig. 20B**) showed significant reduction of interaction time in the recognition phase. However, in the Off-DOX session, neither the control mice (Sti: 38.37 ± 3.455; Fam: 32.82 ± 4.796; paired *t* test: $p = 0.144$;n = 31. **Fig. 20C**) nor the CA3-TeTX mice (Sti: 41.47 ± 3.833; Fam: 35.99 ± 4.19; paired *t* test: $p = 0.180$; n = 42. **Fig. 20D**) showed significant reduction of interaction time in the recognition phase. Moreover, The SRM index among each group did not show any statistical difference (Ordinary one-way ANOVA: $F(3, 120) = 0.6091$; $p = 0.610$). I firstly speculated that this general reduction of interaction in the sampling phase might be because of the food switch. To verify this possibility, I tested a new cohort of CA3-TeTX mice,

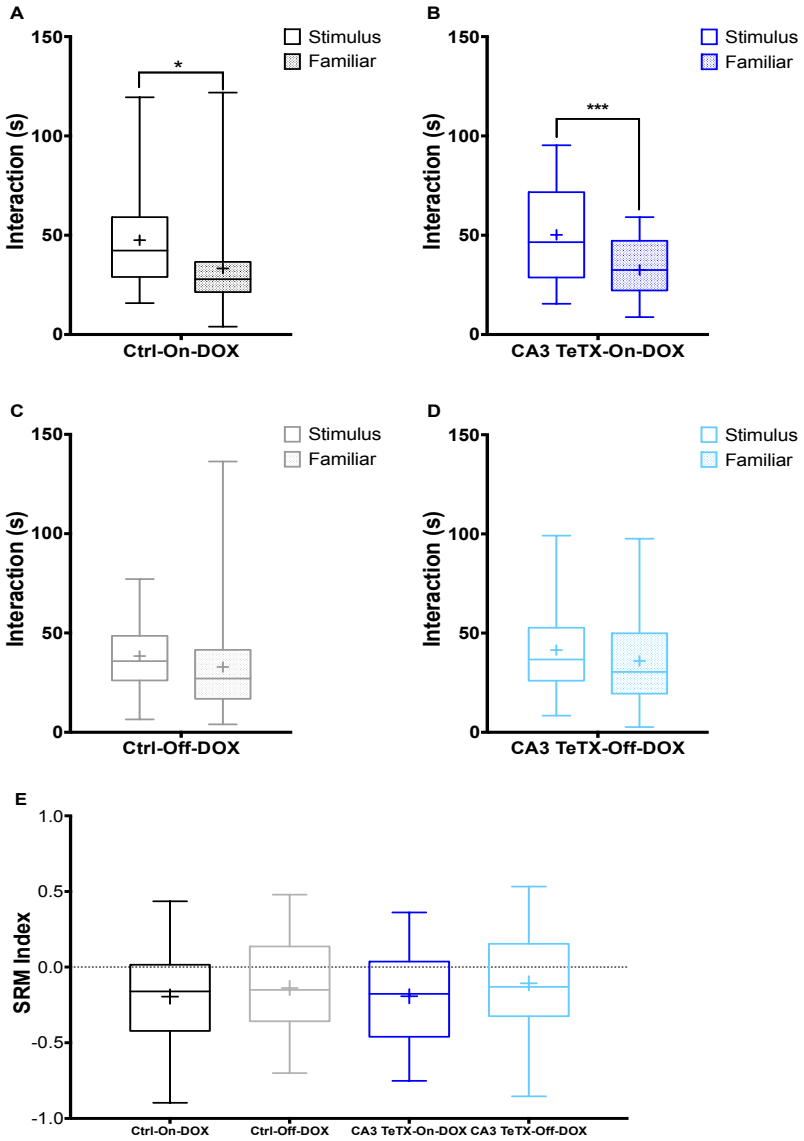


Figure 20. DOX food withdrawal affects social recognition memory. Interaction time toward the same social opponent (A) Control group on DOX session. Sti: 47.48 ± 4.191; Fam: 33.28 ± 4.315; paired *t* test: $p = 0.022$; n = 31. (B) CA3-TeTX on DOX session. Sti: 50.19 ± 4.366; Fam: 32.51 ± 2.668; paired *t* test: $p < 0.001$; n = 31. (C) Control group off DOX session Sti: 38.37 ± 3.455; Fam: 32.82 ± 4.796; paired *t* test: $p = 0.144$; n = 31. D CA3-TeTX off DOX session. Sti: 41.47 ± 3.833; Fam: 35.99 ± 4.19; paired *t* test: $p = 0.180$; n = 42. (E) SRM index of different KO animals. (Ordinary one-way ANOVA: $F(3, 120) = 0.6091$; $p = 0.610$.)

where the transgene is controlled by DOX water (10 mg/kg DOX in 1% sucrose water), to eliminate the unknown factors during food switch. Following the same behavioral test paradigm, again both control mice (Sti: 36.89 ± 4.3; Fam: 17.7 ± 1.5; unpaired *t* test: $p = 0.0034$; n = 7. **Fig. 21A**) and CA3-TeTX mice (Sti: 36.75 ± 5.6; Fam: 16.3 ± 2.2; unpaired *t* test: $p < 0.001$; n = 11. **Fig. 21B**) showed significant reduction of interaction time in the recognition phase in the On-DOX session. Unexpectedly, again, neither the control mice (Sti: 23.67 ± 6.6; Fam: 21.71 ± 3.0; unpaired *t* test: $p = 0.7963$; n = 7. **Fig. 21C**) nor the CA3-TeTX mice (Sti: 17.35 ± 2.3; Fam: 14.47 ± 2.6; unpaired *t* test: $p = 0.5057$; n = 11. **Fig. 21D**) showed significant reduction of interaction time in the recognition phase in the Off-DOX session, which suggested that although the CA3-TeTX transgenic line is a useful system for spatial memory research, this chronic output transmission blockade might not be a good animal model for the tasks involving interactions between individuals. I will address this problem in the discussion.

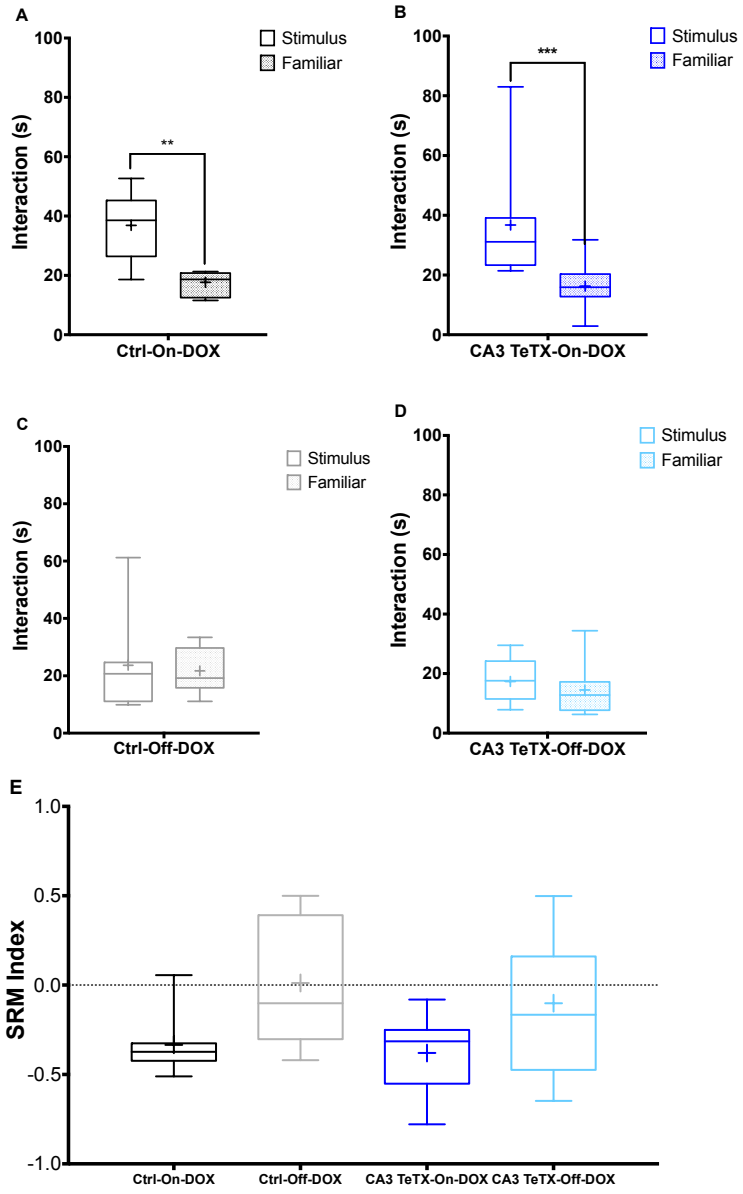


Figure 21. DOX withdrawal affects social recognition memory. Interaction time toward the same social opponent (A) Control group on DOX session. Sti: 36.89 ± 4.3; Fam: 17.7 ± 1.5; unpaired *t* test: $p = 0.0034$; n = 7. (B) CA3-TeTX on DOX session. Sti: 36.75 ± 5.6; Fam: 16.3 ± 2.2; unpaired *t* test: $p < 0.001$; n = 11. (C) Control group off DOX session. Sti: 23.67 ± 6.6; Fam: 21.71 ± 3.0; unpaired *t* test: $p = 0.7963$; n = 7. (D) CA3-TeTX off DOX session. Sti: 17.35 ± 2.3; Fam: 14.47 ± 2.6; unpaired *t* test: $p = 0.5057$; n = 11. (E) SRM index of different KO animals. (Ordinary one-way ANOVA: $F(3, 32) = 3.425$; $p = 0.029$; Tukey's multiple comparisons: Ctrl group on DOX vs. off DOX: $p = 0.149$; CA3-TeTX on DOX vs. off DOX: $p = 0.142$.)

Social hierarchy was not affected by CA3 activity

In the previous sections I have found that manipulation of CA3 plasticity and activity affects SRM in a short time interval manner. My next question was whether CA3 activity is also involved in long term SRM. A good candidate of long term SRM is the memory of siblings, housed in the same cage after weaning. Normally grouped house mice live peacefully in the same space because of the existence of social dominance structure called social hierarchy, which is common among the social animals and is consistent and stable over time[65]. Once the social hierarchy is established, the individuals can minimize the aggressive behavior among other members. Therefore, I hypothesized that the long-term SRM is the cornerstone for stable social dominance. If I perturb long-term SRM, I can observe the social hierarchy collapse, then I can test the involvement of CA3 in long term SRM by observing the stability of social hierarchy. To address this hypothesis, I applied the well-established experimental approach, tube-test. To test the memory effect of social hierarchy, I selected CA3-TeTX animals as subjects since the transgene is inducible by antibiotics and can chronically express after the induction. By using these transgenic mice, I can compare the social hierarchy before and after the TeTX expression; also, I can measure the stability of social hierarchy during the TeTX induction period. I tested every paired-interaction combination of mice of each cage. For example, 5 mice give 10 different pair of match combination, the match result was recorded and the subject who won most was assign to RANK 1. Blue line and red line indicate the social ranking of CA3-TeTX mice and controls, respectively. I did not observe the consistent trend of in-cage ranking fluctuation among three cages (**Fig. 22**). I also tested the acute inhibition of CA3 activity during the dominance test in dorsal CA3-DREADDs animals (**Fig. 23**). Blue line and red line represent social ranking of the CA3-hM4d(Gi) mice and CA3-mCherry mice,

respectively. Again, in the acute manipulation of CA3 activity seemed having no effect on the social ranking. I will also discuss this issue in the discussion.

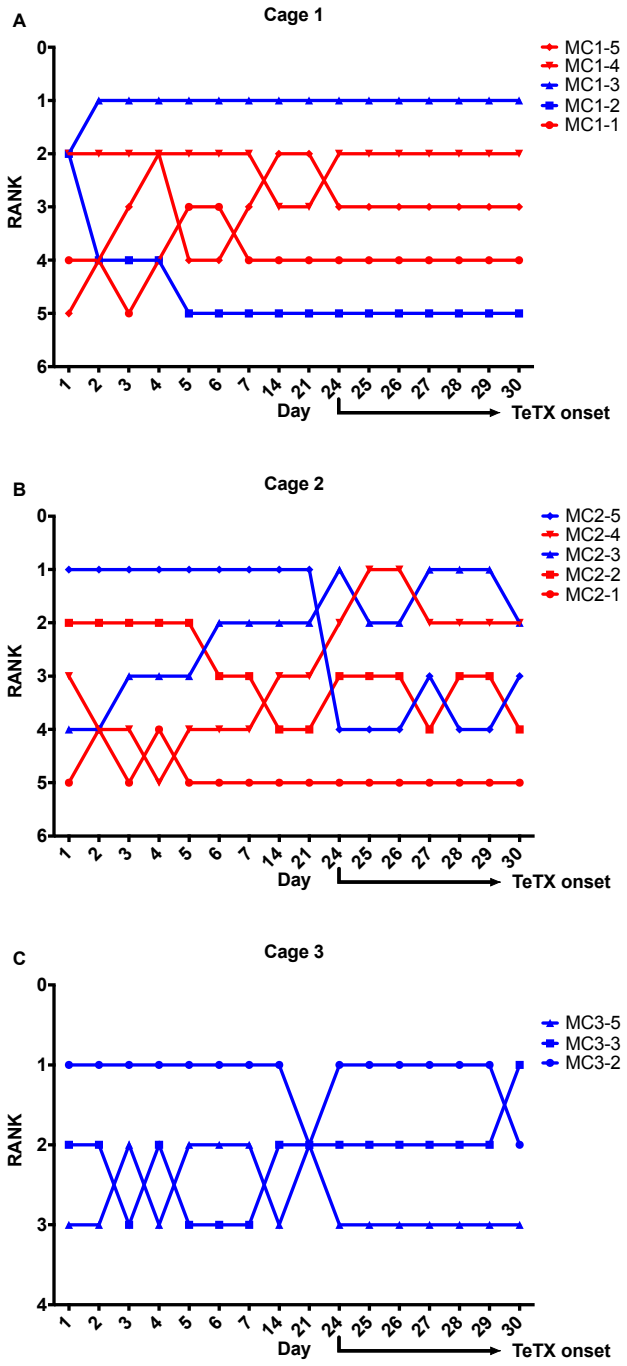


Figure 22. In-cage social ranking of CA3-TeTX mice.

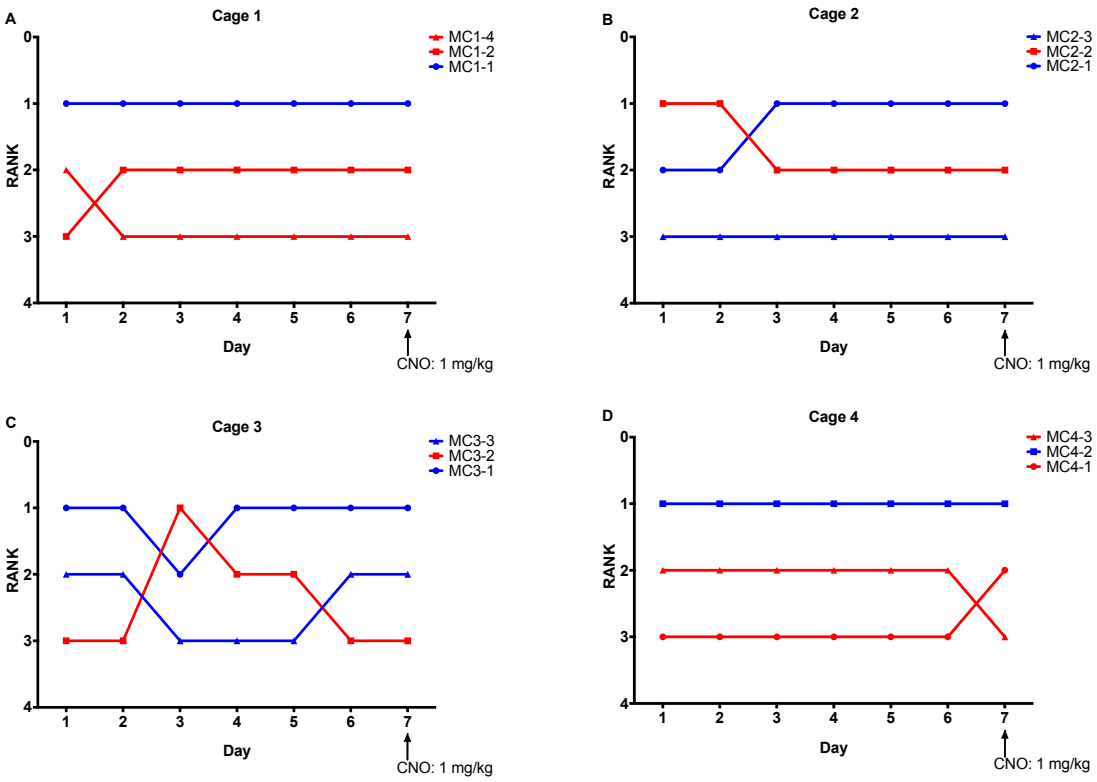


Figure 23. In-cage social ranking of dCA3-DREADDs mice.

Alternative statistical analysis of behavior results

For my data analysis, I compared the difference of the raw exploration time and normalized exploration indices among groups by several statistical approaches, including paired and unpaired *t* test and ANOVA test. However, it has been suggested that the classical statistical approaches do not provide direct evidence for inference and an alternative Bayesian inference provides more information for group comparison. Therefore, I'd like to test this idea whether the Bayesian inference can give me more confidence in my behavioral research. I used Python language and the PyMC3 package for Bayesian inference. I first focused on the comparison of the SRM indices between control and CA3-NR1 KO animals. The central idea for Bayesian inference is to set priors for later inference. I therefore set a normal distribution derived from the experimental mean and standard deviation as the prior distribution of group mean and the degree of freedom to 30 which makes the *t* distribution almost identical to the normal distribution. I next applied the No-U-Turn Sampler (NUTS), an alternative Markov Chain Monte Carlo (MCMC), methods for the posterior sampling, and then plotted the posterior distribution (**Fig. 24**). The difference of mean was -0.157 with 95% highest posterior density

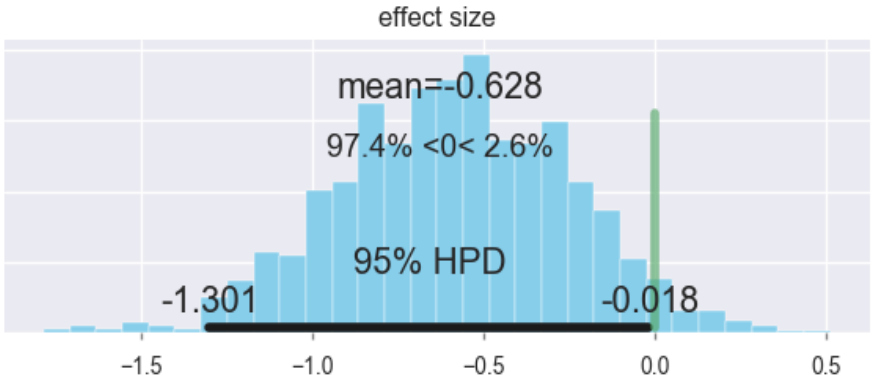
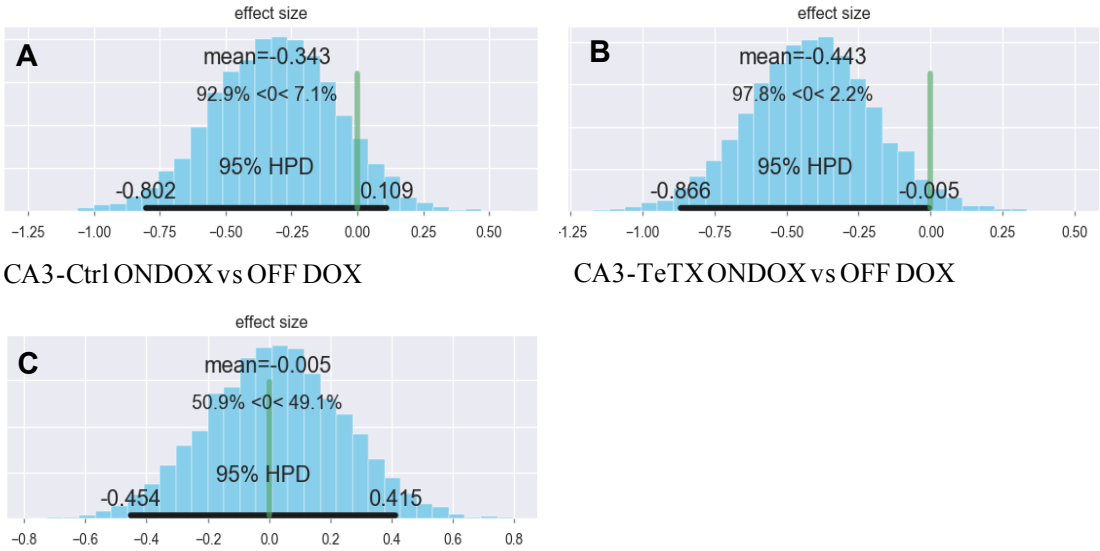


Figure 24. Bayesian estimation of CA3-NR1 KO SRM index.

(HPD) ranged from -0.304 to 0.005. The effect size between two groups is -0.628 with 95% HPD ranged from -1.301 to -0.018. Although the 95% HPD contains 0, the estimation of

difference of mean indicated that 97.4% of posterior are lower than 0. In addition, the 95% HPD of the effect size didn't contain 0, which also suggested that the SRM indices of CA3-NR1 KO animals are different from the control animals. Therefore, we have higher confidence to claim that the CA3-NR1 KO did affect animals' social recognition memory. The same estimation was performed for CA3-DREADDs experiments (**Fig. 25**) and for CA3-TeTX



CA3-Ctrl ONDOX vs OFF DOX

CA3-TeTX ONDOX vs OFF DOX

CA3-Ctrl OFFDOX vs CA3-TeTX OFF DOX

Figure 25. Bayesian estimation of CA3-TeTX SRM index.

experiments (**Fig. 26**). I found similar results as the classical statistical analysis that the ventral CA3 CNOI group demonstrated the strongest effect (effect size= -1.576), where the 95% HPD of both effect size and the difference of mean exclude the null value (0). In the rest of group comparison where the effect size of zero falls in the 95% HPD, which means there are some uncertainty in the estimation.

Together, by using an alternative approach for the behavioral performance inference, I can make the same conclusion with higher confidence that hippocampal CA3 plasticity is essential for social recognition memory; furthermore, the integrity of ventral CA3 activity during social memory encoding is essential for successful familiar recognition in the near future.

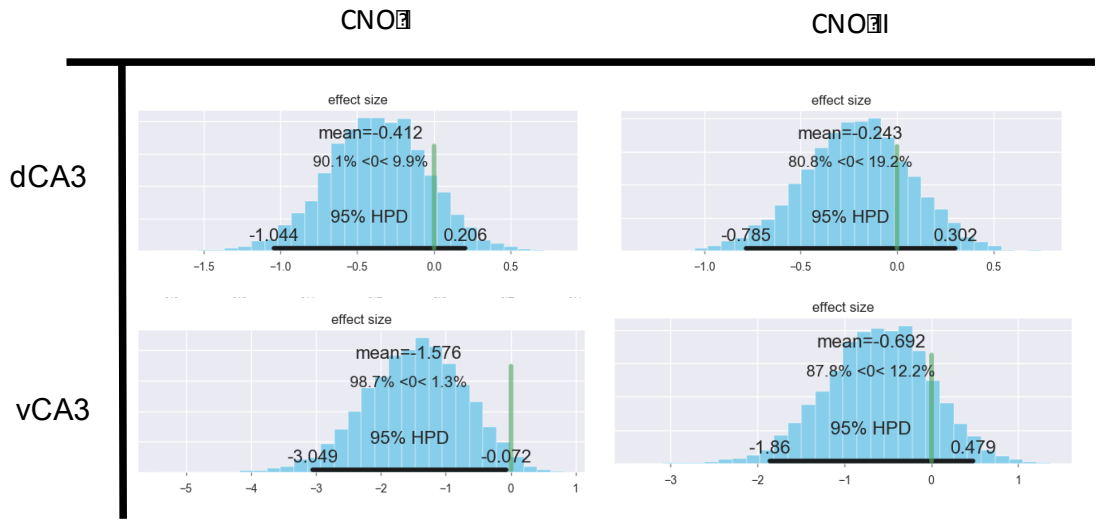


Figure 26. Bayesian estimation of CA3-DREADDs SRM index.

Chapter 4 Discussion

Intrahippocampal network for social memory

Here I find that hippocampal CA3 pyramidal cell synaptic plasticity and activity are both necessary for social recognition memory (**Fig. 9C, 18A**), while plasticity in the DG is dispensable (**Fig 9D**). Moreover, taking advantage of the spatial and temporal control of the DREADD system, I refine my findings to demonstrate that dorsal CA3 is dispensable for SRM (**Fig. 16**), while ventral CA3 is required for the encoding, but not the recall of social stimuli (**Fig. 18**). These data reaffirm the functional heterogeneity across the dorsal-ventral axis of hippocampus[45] and are in line with the associative encoding functions of CA3[39].

Differential task specific engagement of plasticity in specific hippocampal subregions has been demonstrated in both spatial and non-spatial tasks. Here I assess how these interventions impact social recognition memory by taking advantage of an animals' innate drive to explore a novel conspecific more than a familiar one. Natural social behavior across species is based on previous experience, thus may involve the social component of episodic memory. This suggests that, like spatial and contextual models of hippocampal function, rodent social memory is based on the comparison of current social stimuli with prior social interactions. In contextual memory CA3 plasticity has been demonstrated to be crucial for rapid associative coding that can later support recall via pattern completion mechanisms[30, 51], while DG plasticity supports context discrimination via pattern separation[29]. Interestingly DG-NR1 KO mice only demonstrated an impaired response to a novel context if they have previously experienced similar, yet distinct environments[21, 29]. Here I find that these mice behave similar to control mice in both social memory and social novelty recognition under my standard protocols. Given possible parallels with contextual memory, future work addressing the experience dependent aspect of these

behaviors may reveal that after increased social experience a pattern separation-like function of DG plasticity may be required. The impairment in SRM I observed in the CA3-NR1 KO mice suggests deficits in encoding in the recurrent CA3 network. This again would parallel the phenotypes observed in contextual and spatial tasks with these mice[21, 30, 59], and support the hypothesis that social stimuli are associatively encoding in an episodic-like manner.

The afferents and efferents along the long dorsal/ventral (d/v) axis of the hippocampus are distinct and the anatomy supports a functional distinction, with dorsal hippocampus more involved in spatial memory and ventral in emotional and anxiety-related responses[17]. In human fMRI studies differential activation can be observed along the long axis of the hippocampus depending on the type of stimuli presented, with visual information preferentially activating posterior (dorsal) hippocampus and olfactory or auditory information activating anterior (ventral) regions[66]. Interestingly while unfamiliar faces activate the more posterior regions, pairing those faces with names, perhaps giving them additional social context, leads to a shift to activation in more posterior regions[66]. Previous work on the CA3-NR1 KO mice demonstrated an odor specific experience-dependent shift in hippocampal activity along the d/v axis requires CA3 NMDA receptors[31], presumably due to the recurrent connectivity that exists along this axis. Given that olfaction is the dominant sensory modality by which rodent social information is communicated[67], this suggests the vCA3 may be involved in the initial processing and encoding of social aspects of episodic memory. My acute DREADD inhibition experiments support this interpretation, with dorsal CA3 dispensable but ventral necessary for the social stimuli encoding. Further, given that the primary target of vCA3 outputs are vCA1 pyramidal cells, together with a recent work demonstrating a similar gradient in CA1[15] this

suggests functional specialization across the d/v axis.

Recent work has highlighted the unique physiology and behavioral contributions of the CA2 subregion of the hippocampus[12, 14, 21, 23, 24]. Chronic silencing[12] or lesions[13] of dCA2 specifically impair social recognition memory. This specialization may be related to inputs from the hypothalamus[25, 68], which can regulate CA2 excitability via release of vasopressin. Interestingly, manipulations which locally alter the inhibitory network in CA2 also can result in social memory deficits[69, 70], suggesting the inhibitory/excitatory balance in this region may be a key regulation point of hippocampal-mediated social behavior. The axons of dorsal CA2 pyramidal cells show extensive projections along the dorsal/ventral axis, targeting both ventral CA3 and ventral CA1[15, 26]. Understanding how these CA2 inputs are involved in regulating the encoding of social memory in these ventral areas will be an interesting next step in assembling the functional hippocampal network activated by social stimuli.

Extrahippocampal networks for social memory

While the hippocampus clearly plays a role in social memory, it is important to consider where it sits in the larger networks of the brain controlling social behavior. Anatomy suggests that the hippocampus may sit between the olfactory system for social stimuli perception and the hypothalamus for behavioral control. The anatomical connections relaying olfactory information into the hippocampus and the physiological information relay between these two regions have been determined[7, 8]. Olfactory input is relayed to the hippocampus primarily via the superficial layers of the central lateral entorhinal cortex, which preferentially targets the ventral hippocampus[71]. Moreover, one evidence has also suggested with pharmacological and optogenetic manipulation of the basolateral complex of the amygdala (BLA) projections to

ventral hippocampal CA1, social behavior is modulated bidirectional[72]. A more recent work on the medial amygdala (MeA) further demonstrated the neuronal representation of social information in behaving mice[73]. In addition, other subcortical inputs preferentially target the ventral hippocampus, including the interpeduncular nucleus (IPN)[74]. Neurons in the IPN reduce their activity in response to familiar social stimuli[75], suggesting a convergence of multiple signals into this region. Social information which is processed by hippocampus then is sent to downstream areas of the brain. The medial and ventral portions of the hippocampus preferentially target the ventral portions of the lateral septum, which relay information to the hypothalamic regions involved in social actions[17]. Recent research has provided evidence that neural representations in mouse hypothalamus that underlie social behaviors are shaped by social experience[76]. Besides the hippocampal-septal-hypothalamic relay, the ventral hippocampal-nucleus accumbens (NAc) circuits are suggested to have social memory engrams[15] and recent research further expended the idea that the projection from prelimbic cortex (PL) to NAc combined the social and spatial coding and supported social-spatial learning[77].

In summary, current data have suggested a network for social memory representation and social behavior modulation. It is important to understand the precise information flow and processing across these different brain regions with excellent temporal resolution. However, current techniques have limitations which make this challenging. For example, extracellular electrophysiological probes have excellent temporal resolution but lack wide coverage. While Ca^{2+} imaging has greater cell coverage but lacks temporal resolution. Therefore, more advanced tools for neuroscience research are needed and recently developed silicon probes might able to

meet this need[78].

The repertoire of social recognition

In my social recognition memory test, I reduced the range of social recognition to only comparison of the conspecific male-male recognition. However, the repertoire for natural social recognition memory is not limited to conspecific male-male recognition, it also includes male-female, female-female and cross-species recognition. It has been reported that intact male mice interact with conspecific more than female[79], thus whether the manipulation of hippocampal CA3 can also impair the exploration difference between female-female or cross-sex social recognition requires further investigation. A caveat for cross-sex social recognition task is that the direct interaction paradigm for male-female might cause sexual behavior during the test; therefore, the only way to address the male-female social recognition is applying the social choice task, in which the test protocol has been well established[80].

The unsolved questions in CA3-TeTX mice social behavior

In my present studies, I have demonstrated the social recognition impairments were related to CA3 plasticity and activity. However, the experiments of the social recognition memory with CA3 transmission blockade did not provide conclusive information because of the DOX switching process affecting control animals' social recognition performance. As I mentioned in the result section, I first speculated the behavioral change in the controls is due to the food change. Since the DOX food and normal food for animals are come from different companies; it is impossible to precisely control the food content under current condition. To address the problem, I

prepared a new cohort of CA3-TeTX mice which were fed with DOX-water and normal food. In this experimental design, I can induce the TeTX expression by removing the DOX exclusively from water without changing any other nutrition contents, which can minimize the food-switching effect on the animal behavior. However, the SRM impairment was still observed in the control animals, which indicated another internal factor changes the behavior of control animals. With a more careful animal monitoring, I noticed that after long term (more than two months) DOX withdrawal, animals showed intra-cage aggressive behavior. Regardless of the number of animal in the cage, one aggressive individual attacks his siblings fiercely. This finding suggested that except the aggressive individual, the rest of animals in the cage suffered from the chronic social defeat stress. It has been reported that animal with adulthood chronic social defeat stress reduces social motivation and social interaction[2]. The feeding condition for my CA3-TeTX mice is that regardless the genetic background animals are grouped house together and after the SRM test the behavioral performance were then grouped by their genetic background. Therefore, the impairment in SRM task of my control animals might be because of the chronic social defeat stress in the home cage. An additional important observation is that all the aggressive individuals are CA3-TeTX transgene positive mice. However, whether this aggressive behavior in CA3-TeTX mice is directly because of the dysfunction of CA3 output or is the general aggressive level elevation requires a more careful experimental design to address.

Statistical inference for behavioral results

Reproducibility in scientific research has always been an important issue. However, the

growing number of studies have indicated that incorrect statistical usage[81] and low power statistics contribute to the irreproducible results in different science disciplines. In neuroscience, on average, the statistics used in studies were under power across the fields[82, 83]. Moreover, the statistical significance test only reported that whether the two groups are different or not, it did not provide any information of the magnitude of the difference between groups. Therefore, reporting the *p* value might not be enough to form a complete conclusion from the experimental observation, and indicating the effect size should be considered[84]. Moreover, the effect size is an essential parameter to calculate the sample size based on the required statistical power (priori power analysis) or to calculate the statistical power based on the given sample size (posterior power analysis)[62, 85]. In addition to the classical frequentist statistics, the Bayesian estimation for group difference has also been suggested. Therefore, in my studies, I ran both analyses for better data inference.

Hippocampal dysfunction in psychiatric disorder

Social behavior deficit is pervasive in major neuropsychiatric disorders, such as schizophrenia. However, the efficacy of existing treatment is still limited; moreover, the neurological mechanism underlying this disease is still enigma. Although the obstacle in clinics, the growing evidence has provided a heuristic model which indicated the NMDA receptor dysfunction induces the social preference disruption[86-88]. In agreement with these studies, my results also showed social memory deficits in NR1-KO mice. In addition to the molecular basis for the schizophrenia, several studies have demonstrated that hippocampal CA2 is affected in schizophrenic patients' postmortem tissue. Consistently, evidence has demonstrated that the

dysfunction of hippocampal CA2 caused social memory deficit[12, 13, 54]. The hippocampal CA2 receives CA3 regulation directly and indirectly (through PV+ interneurons)[22], this inter-wired subunit may explain my findings in which manipulation of CA3 plasticity, transmission and activity cause social memory deficit.

In conclusion, although the detailed neural mechanisms underlying hippocampal social recognition memory still require further elucidation, my data update the current models to demonstrate a specific role of ventral CA3 in the encoding of social stimuli.

Chapter 5 Future Perspectives

As mentioned in the discussion section, my current results neither provide the neural mechanism of CA3 in social memory processing nor the effect of CA3 on CA1 neurons affects social information representation. Moreover, it is still unclear whether the CA3 plasticity and activity are also essential for female-female recognition, cross-gender recognition or long-term social recognition. Last but not least, the relationship between the aggressive phenotype in the CA3-TeTX transgenic mice needs a careful experimental design to be fully understood. Therefore, the future plan following my current results can be separated to three main directions: **1.)** To determine the *in vivo* physiological properties during the social encoding in ventral hippocampus. **2.)** To test the involvement of hippocampal function of full repertoire of social recognition memory, in terms of cross-gender recognition and long-term recognition. **3.)** To test whether the blockade of CA3 transmission increases general aggression level or causes sibling recognition deficit.

Elucidating the hippocampal physiology for social memory

The physiological properties of hippocampal principle neurons have been well determined during spatial navigation and spatial learning. Moreover, recent studies also indicated hippocampal activity changed during social behavior. Previous research in ventral CA1 and my current results in ventral CA3 suggest that ventral hippocampus plays an important role in social memory. However, none of the research demonstrates the ventral hippocampal physiology during social interaction. To address this question, simultaneous recording of neural activities from vCA3 and vCA1 during social behavior is required. By comparing the spike timing, local field potential oscillation, and spike-oscillation coupling during social behavior, these results

may help to correlate the neural activity to behavior performance. With the further optogenetic perturbation during behavior, we may be able to build a model for social memory processing form the recorded neural activity.

Expanding the hippocampal-dependent social recognition repertoire

In my present work and much of the related literature, the social recognition task is restricted to either male-male familiar recognition or male-male novelty recognition. The recognition ability across genders in male mice and the recognition ability of female mice are rarely discussed. The reason for the former might be due to the instinct for mating while the male mouse meets a female mouse and the latter might be due to the less motivated interactions between female individuals. The first question can be addressed by an alternative approach to assess the social recognition memory by social choice paradigm[80]. In my current results I have tested male CA3-NR1 KO mice for female recognition **(Fig. 27**), the sociability is intact in both controls and CA3-NR1 KO mice (Two-way RM ANOVA: Genotype × Social stimulus $F(1, 19)$

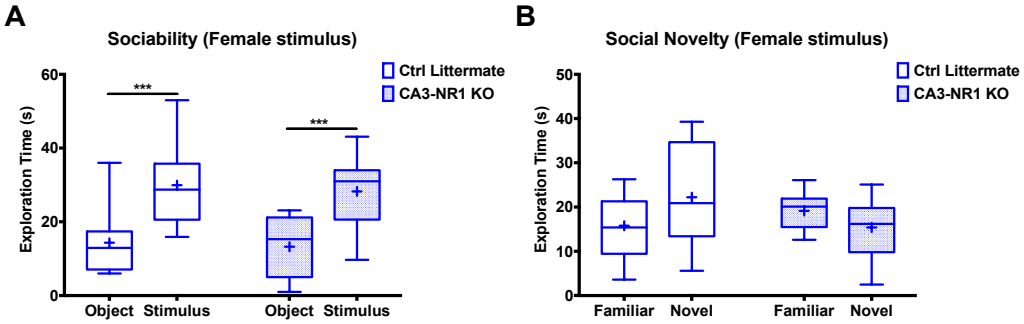


Figure 27. Social choice test of CA3-NR1 KO mice show social novelty impairment with female stimulus. (A) Sociability session. (Two-way RM ANOVA: Genotype × Social stimulus $F(1, 19) = 0.02218$, $p = 0.886$; Genotype: $F(1, 19)$: 0.1509, $p = 0.702$; Social stimulus: $F(1, 19) = 53.49$, $p < 0.001$. Multiple comparison of object vs. Stimulus with uncorrected Fisher's LSD: Ctrl: $p < 0.001$; CA3-NR1 KO: $p < 0.001$). (B) Social novelty session. (Two-way RM ANOVA: Genotype × Social stimulus $F(1, 19) = 6.499$, $p = 0.02$).

= 0.02218, $p = 0.886$; Genotype: $F(1, 19)$: 0.1509, $p = 0.702$; Social stimulus: $F(1, 19) = 53.49$, $p < 0.001$. Multiple comparison of object vs. Stimulus with uncorrected Fisher's LSD: Ctrl: $p < 0.001$; CA3-NR1 KO: $p < 0.001$; **Fig. 27A**) and CA3-NR1 KO mice showed impairment in social novelty test (Two-way RM ANOVA: Genotype × Social stimulus $F(1, 19) = 6.499$, $p = 0.02$; **Fig. 27B**), which captured the same behavioral deficit of the social choice test with male mouse stimulus (**Fig. 11**). These results expanded my conclusion of the CA3 plasticity for social recognition memory, in which the CA3 plasticity is required for both the male and female recognition. Therefore, by using this female stimulus social choice paradigm, it is important to test the requirement of CA3 activity for female social information encoding. The social recognition memory in female mice will follow the same behavioral test protocol to assess the memory performance.

Understanding the aggressive phenotype in CA3-TeTX mice

To verify the cause of aggressive behavior in CA3-TeTX mice, it is important to confirm that this aggressive behavior is exclusively induced by CA3-TeTX transgene expression and not related to the DOX food switch. A negative control for this intra-cage aggression test is needed, where the transgene negative littermates undergo the DOX food withdrawal. Health condition is monitored after 2 month DOX withdrawal. The working hypotheses behind this aggressive behavior are: **1.)** The CA3 transmission blockade causes the elevation of general aggression level. **2.)** The CA3 transmission blockade causes the impairment of siblings' recognition, therefore the transgene positive individual treats same cage sibling as intruder and then initiates aggressive behavior. To address these questions, the basic understanding for aggressive behavior[89] is needed and recent research on the mouse aggressive behavior[90] provides useful information for my future research on this fierce aggressive phenotype in CA3-TeTX mice.

In conclusion of my future perspective, these three topics are interesting projects all of which need careful experimental design. Regarding the experimental design with consideration of the recent problem in neuroscience research, the average statistical power in neuroscience studies is low[82], and with the inspirations form two neuron perspective articles[91, 92]. A framework which adapts a new statistical analysis for neural activity recorded during behavior will help me to determine whether the specific neural activity is involved in behavioral tasks. Therefore, to build a strong statistics knowledge and programming proficiency for the in vivo physiology data analysis will become the primary lessons for me during the project execution periods.

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List of research achievements

As of February 2018

Journal articles

1. **Ming-Ching Chiang**, Arthur J Huang, Marie E. Wintzer, Toshio Ohshima, and Thomas J McHugh. A role for hippocampal CA3 in social recognition memory. Behav. Brain Res., (2018), doi: 10.1016/j.bbr.2018.01.019. **(*In press*)**
2. Lily M. Y. Yu, Denis Polygalov, Marie E. Wintzer, **Ming-Ching Chiang**, and Thomas J. McHugh. CA3 Synaptic Silencing Attenuates Kainic Acid-Induced Seizures and Hippocampal Network Oscillations. eNeuro, ***3(1)***, 0003-16.2016, 1-18 (2016), doi: 10.1523/ENEURO.0003-16.2016

Conferences

1. **Ming-Ching Chiang**, Arthur J Huang, Roman Boehringer, Denis Polygalov, Toshio Ohshima, Thomas J. McHugh. Differential contributions of dorsal and ventral CA3 to social memory. Neuroscience 2017, (Poster 426.11) Nov., 2017.
2. **Ming-Ching Chiang**, Arthur J Huang, Toshio Ohshima, Thomas J. McHugh. Hippocampal CA3 plasticity and transmission are required for social memory. Neuroscience 2015, (Poster 725.05-We) Oct., 2015.

Acknowledgement

At first, I would like to express my gratitude to my family who fully supported my life in Japan without any hesitation. I also want to thank Professor Ohshima who cares my research progress more than I do. Without his regular supervision, I will not be able to finish my doctor program within 4 years. Next, I'd like to thank Professor Inoue, without his suggestion and critical comment on my statistical inference, I will never consider the importance of statistics approaches and the precision of my data interpretation carefully. Last but not least, I would like to express my gratefulness to my advisor Dr. McHugh. Thank you for accepting me to be your first full-time Ph.D. student; thank you for allowing me to join your lab when I don't have sufficient background knowledge in neuroscience; thank you for forgiving me when I made mistakes and no progress during the training period. Most importantly, thank you for giving me an environment where I can ask stupid questions, learn from experienced researchers, and develop my own research skills for future. I do learn a lot from you and your philosophy in science. I'm sure I will keep these spirits and pass them to the future comers as great legacies. I also want to thank all members in CBP, Roman, Art, Marie, Denis, Kaz, Steve, Shuo, Adam, Hongshen, Hefei, Eriko, Frank, Rachel and especially Michiko-san. Without your help and support, I can't finish my project smoothly. Finally, I would like to thank all my friends. I can't imagine my life without your participation. Thank Li-Feng and Bruce, you two are my best companion both in science and life. Please let us keep in touch forever. There are still too many people whom I want to thank, but please forgive me if I did not mention your name explicitly. Thank you!