Hippocampal microcircuits for social memory specification

By
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Abstract

During social interactions, humans and social animals can distinguish not only familiar and novel conspecifics (social recognition) but also between multiple familiar individuals (social specification). Recent studies have implicated hippocampal sub-region dorsal CA2 (dCA2) in social recognition and identified social recognition memory engram in downstream ventral CA1 (vCA1). However, the anatomical site for the storage of social specification memory and its underlying neuroscientific mechanisms are poorly known. Here, we report that social specification memory engrams are stored in vCA1 while social information encoded in dCA2 becomes sharpened as it travels from dCA2 to vCA1 microcircuits within CA2, thereby acquiring a progressive increase in specification through repeating motifs of feed-forward inhibition. Both the inhibition of GABAergic inhibitory neurons in CA2 and reduced activity of excitatory neurons by ablation of oxytocin receptors in the dCA2 to vCA1 microcircuits impair social memory specification. These results suggest that the vCA1 and the multiple feed-forward inhibition motifs in the dCA2 to vCA1 microcircuits are crucial for social memory specification.

Thesis Supervisor: Susumu Tonegawa

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This thesis is dedicated to

my parents, Tat Ghee Lim and Seo Tee Ong,

my brothers and sister, Osel Shaofeng Lim, Stacey Yiwei Lim and Arnold Weiyu Lim,

my fiancé, Chun Kiat Yeo

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Chapter 1. Literature

1.1 Social memory

In a world where creatures roam, in solitude or in groups, they share ecosystems in which they cohabit, compete for resources, and reproduce. Naturally, one of the processes that is vital to their survival is that of social memory (Kogan JH et al., 2000). Social memory constitutes a span of cognitive and behavioral processes which give rise to a plethora of abilities such as recognizing a "friend" from "foe", familiar from novel conspecifics (commonly termed as social recognition), distinguishing several within-group familiar conspecifics, the ability to learn from conspecifics as seen in foraging (commonly termed as social learning), establishing social hierarchies, mate selection, mate and offspring recognition, establishing and defending territories, and for the general maintenance of the social group (Ferguson JN et al., 2002; Jacobs SA et al., 2016).

For many eusocial species, including humans, navigating through the social world via engaging in meaningful relationships and eliciting the appropriate behavioral responses implies a reliance on the effective recall of individual identities. Species spanning from creatures as large as elephants and dolphins to as tiny as mice and paper wasps, and higher-order social beings like humans, demonstrate the capacity to remember familiar conspecifics – ranging from an inter-trial interval of a week for paper wasps to decades for larger mammals like elephants, dolphins and humans.

1.1.1 Insects

In the realm of insects, *P. fuscatus* (i.e. paper wasps) nest founding queens are classified as organisms with complex, individually differentiated social relationships that rely heavily on individual recognition of varying visual features unique to particular individuals (Tibbetts EA, 2002) (Figure 1). As the founding queen wasps may establish nests with a group of other queens, they will first battle to put the social hierarchy in place before cooperating to build a nest (Reeve, H.K., 1991). Hence, it is ecologically efficient to remember prior social interactions and/or battles with each queen wasp to avoid unnecessary energy-expensive contests. Presented evidence showed that memories of past interactions with a particular conspecific are robust to decay over an inter-trial interval of a week, as well as to interference from subsequent social interactions with ten other individual wasps (Sheehan MJ et al., 2008). The *drosophila melanogaster* seems to also exhibit the ability to distinguish between individual conspecifics. It has been demonstrated that, given a 30-minute separation, flies not only discriminated familiar from

novel opponents, they also recalled the social hierarchy established during the last encounter with that opponent (i.e. familiar or unfamiliar winner, familiar or unfamiliar loser) as evident from the fewer encounters during the first 10 min of fighting in familiar pairings than in unfamiliar pairings, and that former losers fought differently against familiar winners than against unfamiliar winners (Yurkovic A et al., 2006). On the contrary, a study that argue against that claimed that the drosophila memory retrieval process is only facilitated in a social group via taking advantage of chemical signals given off by other drosophila during aggregated social interactions (Chabaud MA et al., 2009). Since stressed flies have been shown to release repellant odorants that include carbon dioxide (Suh GSB et al., 2004), it is suggested that these signals serve as alarm signals to other aggregated flies in the vicinity to enhance their attention or motivation to respond. Therefore, for some social insects such as the drosophila, it may be controversial to claim whether they have social memory *per se*; but it is definitive that they benefit from a social-facilitative effect in an aggregated mass with other flies.

Other colonial insects that may also leverage on a similar collective benefit for survival as the drosophila include a diverse species of ants (Frizzi F et al., 2015) and honeybees (Vernier CL et al., 2019) lie on the other spectrum of social complexity as they have been shown to lack a precise degree of social recognition compared to insects like the queen paper wasps. Firstly, ants can only discriminate nestmates from non-nestmates, but not amongst nestmates. Secondly, it is also evident that ants would not turn aggressive on non-nestmates from a neighboring colony but would turn aggressive on non-nestmates from a distant colony. The underlying rationale for such behaviors is that ants do habituate to the “colony odor signatures” of their proximal neighbors over time (Sherman PW et al., 1997; van Zweden JS et al., 2010); hence rendering the degree of their social recognition to be relatively imprecise. A similar recognition trait can be observed in honey bees where guard bees are only able to identify non-colony forager bees as intruders but not non-colony member bees which had other roles (Vernier CL et al., 2019).

Illustration 1. Examples of variable facial patterns used for individual recognition in *Polistes fuscatus*. (Adapted from *Michael J. Sheehan and Elizabeth A. Tibbetts, 2008*)

1.1.2 Mammals – Elephants and Dolphins

Larger mammals like elephants (McComb K, et al., 2001; Rasmussen LEL. 1995; McComb K et al., 2000) and dolphins (Sayigh LS et al., 1999; Mercado E et al., 2010; Connor RC et al., 2000; Connor RC et al., 2006; Bruck JN, 2013) are known to be intelligent animals with persistent long-term social memory of their familiar conspecifics. As elephants are social units that roam freely in wide open spaces, the alpha female in the herd would have to depend on her repertoire of vocalization ranges learnt from her family's calls to distinguish between the members and non-members. Dolphins (Sayigh LS et al., 1999) demonstrated through spectrographicanalyses that signature whistles are acoustically stable formore than 12 years. His results relied on the whistle stability of the dolphins – given that the whistles lack changes, and the fact that dolphins remember each other's signature whistles. This system may be the longest-lasting recognition system known; and using spectrographic analyses, found that signature whistles are acoustically stable beyond 12 years. This robust whistle, coupled with the fact that dolphins are well-versed with pod members' signature whistles, allowed researchers to show that dolphins are capable of identifying and recalling a familiar members' whistles for 15 or more years.

1.1.3 Mammals – Humans

For humans, social memory recall is largely founded on faces as the presented stimuli. Studies have shown that our response to human faces is predominantly distinct and preferred over other classes of objects; and this selective preference becomes more apparent as we age (Frank MC et al., 2009). Researches attempt to explain the phenomenon of selectively attending to human faces with a theory of a "default network" in the brain that drives a series of involuntary cognitive processes: thinking about recent events and projecting future ones which are based on social interactions and involve the theory of mind during periods of inactivity (Buckner RL, 2012). Neuroimaging studies of brain diseases such as Alzheimer's, autism, schizophrenia depression etc., provided evidence of a compromised "default network"; thereby handicapping a wide spectrum of social cognitive abilities, including social recognition, for such patients. The establishment of such a theory – one that conditions to social interactions and theory of mind – bolsters the fact that humans gravitate towards connecting and understanding people above all others. This, in turn, motivated the terms: face verification (commonly used when testing subjects on unfamiliar faces which the individual only has a vague sense of having seen the face somewhere in the past e.g. an unfamiliar acquaintance) (Mandal B et al., 2010), and face identification (commonly used when testing subjects on familiar faces which the individual has both a strong sense of familiarity and is able to identify him/her by the name).

1.1.4 Mammals – Rodents

The world abounds with various species of animals that seemingly display advanced levels of complex social cognition. The existence of some species, like non-human primates, being the closest genetic cousins to humans in terms of evolution, raised questions as to why rodents such as *Mus musculus* and *Rattus norvegicus* are chosen animal models widely used as the focal subjects of research pertaining to cognitive, emotional and social abilities. In terms of the evolution timeline, rodents diverged from humans approximately 75 million years ago; and it is theorized that man shared a common rat-like placental animal with other mammals (O'Leary et al., 2013).

The DNA sequence similarity among rodents and humans is highly conserved; with only approximately 10-20% of genes in humans without a clear orthologue in mice (Chinwalla A et al., 2002). Brain-related genes have also been highly-conserved in terms of their coding sequence as well; except that the evolution of the transcription of these genes has resulted in an excess of newly-evolved genes found in the developing human brain as compared to the mouse brain (Zhang YE et al., 2011).

On the molecular level, an example of evolutionary conservation is shown in the fact that the neuropeptides, oxytocin (OXT) and arginine vasopressin (AVP), are critical for social memory in rodents (Lee HJ et al., 2008; Wersinger SR et al., 2007a; Song Z et al., 2018) – the same neurochemicals that have also been demonstrated to affect similar social tasks in humans (McCall C et al., 2012). Pertaining to understanding and dissecting declarative memory, the key processing hub that cannot be overlooked would be the hippocampus. Beginning with the seminar investigations of the patient H.M., who lost a great extent of his memory when his medial temporal lobe was damaged, and extending to present day experiments involving nonhuman primates and rodents, a colossal amount of research evidence has ascribed memory-related functions to the hippocampus (Scoville WB et al., 1957; Olton DS et al., 1976; Morris RGM, 1981; Squire LR, 1992; Moser MB et al., 1995; Kim JJ et al., 1992; Maguire EA et al., 1997; Squire LR et al., 2001; Pothuizen et al., 2004; Klur S et al., 2009). However, there are those who may contend that findings of hippocampal-dependent memory functions cannot be directly translated across species because there are certain intra-hippocampal projections that are unique to certain species of animals, and that not every species is "wired the same way". For example, the dentate gyrus has been shown to project its axons to the stratum radiatum of the CA2 region in guinea pigs, cats, rats, and mice (McLardy T, 1963; Hirama J et al., 1997; Lavenex P et al., 2009; Kohara K et al., 2014; San Antonio A et al., 2014; Dudek SM et al., 2016); but this projection is seemingly non-existent in human and nonhuman primates as the axonal projections from the dentate gyrus terminates in, what seems to be, CA3a/b region (Lorente de No R, 1934; Dudek SM et al., 2016). This, however, may be attributed to the fact that the CA2 region in humans and nonhuman primates have yet to be defined by clear genetic markers; hence there could exist a human and/or nonhuman primate CA2 comparable to that in mice. Nonetheless, the vast majority of studies are still in agreement that the general connectivity within the hippocampus (especially along the longitudinal axis) is conserved to a certain degree of similarity across vertebrates (Broglio C et al., 2003; Butler AB et al., 2005; Striedter GF, 2005), which can then be used to rationalize experimental observations, like spatial cognition, across species. Rodents, especially mice, have been intensively studied; and it has been shown that they do exhibit degrees of cognition that were once thought to be human-specific: capable of social memories and behavior (Young L et al., 2004; Ferguson JN et al., 2002; D'Cunha TM et al., 2011; Olazábal DE et al., 2006; Ross HE et al., 2009), hippocampal-dependent episodic memory (Wilson AG et al., 2012; Liu X et al., 2012; Jeffery KJ et al., 1993; Moser MB et al., 1998; Morris RG et al.,1986; Bannerman DM et al., 1995), even tasks that involve cognitive biases like the "sunk-cost effect" foraging tasks (Sweis BM et al., 2018), and metacognition as in uncertainty-monitoring tasks (Smith JD et al., 2014; Foote AL et al., 2007). Taken together, this suggests that despite some variation in the cytoarchitecture and intrinsic connectivity

between humans and rodents, the relatively economical rodent model can be deemed well-suited for endeavors in hippocampal-related memory.

1.2 CA2 and its role in social memory

It is common consensus that the hippocampus sustains and maintains the organization and longevity of memories (Halgren E et al., 1985; Squire LR et al., 1984; Teyler TJ et al., 1986). The widely studied types of memory tasks supported by the hippocampus includes, but is not limited to, contextual fear conditioning, auditory fear conditioning, and novel object exploration (Denny CA et al., 2014; Tayler KK et al., 2013;Ramirez S et al., 2013; Nakazawa Y et al., 2016; Khalaf O et al., 2018; Lacagnina AF et al., 2019). These tasks are heavily founded on connectivity theories and information flow through the regions confined in the trisynaptic circuit. The hippocampal Cornu Ammonis (CA) 2 region, is a relative tiny presence as compared to its neighboring CA3 and CA1 counterparts; and was only until recently, remained invisible in the field of hippocampal research. In fact, it was so insignificant that when past decades burgeoned with experiments dissecting and establishing the hippocampus as a crucial brain structure for memory (Scoville WB et al., 1957), it was completely ignored as the general consensus supported the notion of information flow via the trisynaptic path from the entorhinal cortex (EC) through the dentate gyrus (DG), to CA3, before ending in CA1 (a circuitry that commonly delineates almost all hippocampal memory-dependent tasks investigated). Consequently, CA2 remained undiscovered and uninvestigated; and was simply labeled as a site for rapid encoding of information (Nakashiba T et al., 2008), for the formation of stable representations of space (O'Keefe J et al., 1971), or just a "transitional zone" located between CA3 and CA1 (Lorente de Nó R, 1934; Woodhams PL et al., 1993; Ishizuka N et al., 1995; Mankin EA et al., 2015).

Advances in modern scientific techniques have uncovered various anatomical and physiological properties of the previously elusive CA2; and there are now evidence to show that CA2, in fact, serves multiple functional roles within the hippocampus – made possible from a combination of its intrinsic properties, as well as intra- and extra-hippocampal connectivity.

1.2.1 CA2 intrinsic properties

The CA2 region of the hippocampus is delineated by the observation of large pyramidal neurons, devoid of complex spines, which are positive for CA2-specific gene markers such as: proteins α-actinin2 (ACTN2), Purkinje cell protein 4 (PCP4, or known as PEP19), striatum-enriched protein-tyrosine phosphatase (STEP, or known as PTPN5), regulator of G protein signaling 14 (RGS14), transmembrane AMPA receptor regulatory protein γ5 (TARP γ5) (Kohara K et al., 2014; Zhao M et al., 2007; San Antonio A et al., 2014; Mercer A et al., 2007; Lee SE et al., 2010; Shinohara Y et al., 2012; Alexander GM et al., 2016; Wyszynski M et al., 1998).

Parvalbumin-, calbindin 1-, and reelin-positive interneurons are found to be comparatively more abundant in CA2 relative to CA3 or CA1 (Piskorowski RA et al., 2013, Botcher NA et al., 2014). This hints at a predominantly inhibitory network present in CA2 that could tightly regulate CA2 pyramidal neuron firing activity.

Another characteristic of CA2 is that the glutamatergic synapses in the stratum radiatum (SR) are highly resistant to long-term potentiation (LTP) induced by typical stimulation methods (Zhao M et al., 2007; Chevaleyre V et al., 2010; Chang PY et al., 2007; Caruana DA et al., 2012). This is postulated to be attributed to the presence of a strong inhibitory network formed by the combination of various inhibitory neuronal subtypes. Another probable rationale for a largely suppressed excitatory synaptic plasticity might stem from the RGS14 gene and its associated downstream RGS14 protein signaling pathway. It has been shown that mutant homozygous mice lacking the RGS14 gene exhibit LTP at synapses onto CA2 pyramidal neurons (Lee SE et al., 2010; Lee YS et al., 2009); hence demonstrating the repressive nature of the RGS14 protein.

On top of these, arginine vasopressin receptor 1B (AVPR1b) and oxytocin receptor (OXTR) are the two most abundantly expressed G protein-coupled receptors (GPCR) in CA2 that contributes to the excitatory network in the region via enhancing excitatory postsynaptic currents (EPSCs) (Pagani JH et al., 2015).

All in all, these briefly summarizes the key components gating and controlling the excitatory-inhibitory balance in CA2 that seems to hold CA2 in default suppression under normal physiological conditions.

1.2.2 CA2 intra-hippocampal connectivity

CA2 receives input from the Schaffer collateral axons of CA3 pyramidal cells, as well as excitatory input from the mossy fibers of the dentate granule cells in the DG (Lorente de Nó R, 1934; Shinohara Y et al., 2012; Kohara K et al., 2014; Llorens-Martin M et al., 2015). Unlike the CA3 inputs to CA1, CA3 inputs to CA2 recruit strong feed-forward inhibition and showed little plasticity under normal physiological conditions (Zhao M et al., 2007; Carstens KE et al., 2019). On the other hand, the cortical inputs that target the distal regions of the pyramidal cell dendrites are robust, highly plastic, and are able to efficiently generate action potentials (Chevaleyre V et al., 2010; Sun Q et al., 2014) (Figure 2.).

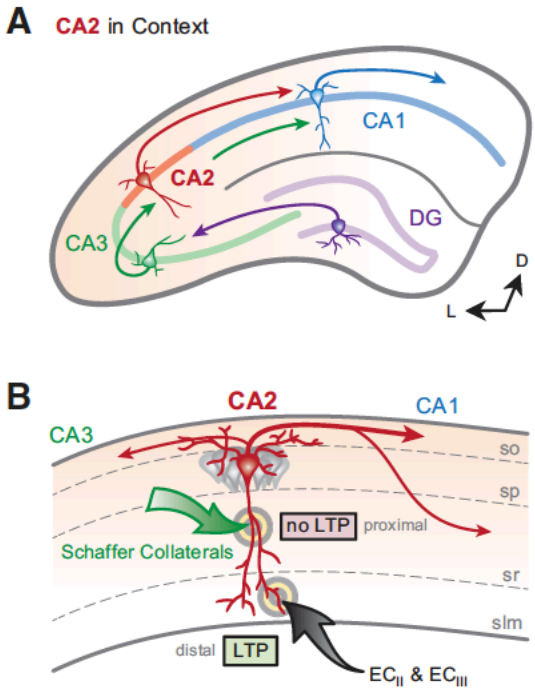

Illustration 2. Depiction of the flow of information through the hippocampus

(A) A simplified schematic diagram showing how CA2 (in red) fits within the traditional "trisynaptic" circuit. Arrows indicate the primary direction of information flow through the circuit.

(B) Induction of activity-dependent LTP differs along the proximal-distal axis of CA2 pyramidal neurons. Schaffer collateral projections to the proximal dendritic compartment of CA2 neurons do not typically support activity-dependent LTP (green arrow). This is in contrast to temporoammonic inputs from the entorhinal cortex to the distal dendrites of CA2 neurons which readily express LTP (black arrow). (Adapted from *Douglas A. Caruana et al., 2012*)

The majority of axonal projections of CA2 pyramidal cells are targeted to CA1 – with high intensity labeling of projection axons in the stratum oriens (SO) that project to deep CA1 pyramidal cells (Shinohara Y et al., 2012; Kohara K et al., 2014) relatively to that in the SR and stratum lacunosum moleculare (SLM) (Cui Z et al., 2013). There are also recurrent collaterals that innervate ipsi- and contralaterally within CA2 (Okamoto K et al.,2019). This could give rise to a concerted effort between the recurrent collaterals and the axons that project back into CA3 to reinforce feed-forward inhibition (Boehringer R et al., 2017); thereby forming the basis of a CA3-CA2 network that can dynamically impact both the flow of information and the excitatory-inhibitory balance of the entire hippocampal circuit (Sekino Y et al., 1997). Dissecting CA2 into dorsal and ventral regions, dorsal CA2 (dCA2) pyramidal cells have been shown to project to all 3 hippocampal CA regions dorsally and ventrally: CA3, CA2, CA1 (Cui Z et al., 2013). This could perhaps rationalize the conventional notion of CA2 functioning as a "transitional" zone for the transmission of information to integrate a memory episode (Moser MB et al., 1995)

1.2.3 CA2 extra-hippocampal connectivity

CA2 also receives inputs from the hypothalamus, namely the paraventricular nucleus (PVN) – a region that contains vasopressinergic and oxytocinergic neurons, which are both crucial in mediating social behavior (Cui Z et al., 2013; Zhang L et al., 2013); via the fornix from the supramammillary nucleus (SUM) – a region found to be involved in oscillatory activity, stress response, novelty detection, conditioned fear and various situations generic for emotional salience (Beck CH et al., 1995; Cullinan WE et al., 1996; Choi WK et al. 2012, Cui Z et al. 2013, Kohara K et al., 2014; Ito M et al. 2009, Pan WX et al., 2002, Wirtshafter D et al., 1998); and entorhinal cortex (EC) layer II – a region which receives both spatial and non-spatial information such as input from the olfactory bulb and piriform cortex for the latter (Chevaleyre V et al., 2010; Jones MV et al., 2011; Caruana DA et al., 2012; Kohara K et al., 2014; Mankin EA et al., 2015; Dudek SM et al., 2016; Boeijinga PH et al., 1984).

CA2 also extends its efferent projections to a myriad of subcortical regions, which could directly or indirectly implicate hippocampal function. It is found that CA2 pyramidal cell projects to the lateral septum (LS) (Cui Z et al., 2013; Leroy F et al., 2018) – a region reported to be strongly influenced by CA2 outputs in promoting social aggression via the CA2-lateral septum-ventrolateral subnucleus of ventromedial hypothalamus (VMHvl) circuitry; as well as the medial septum (MS) and diagonal band of Broca (DBB) (Cui Z et al., 2013) – regions which are coupled to and regulated by hippocampal

oscillatory activity (Bender F et al. 2015, Chee SA et al. 2015, Dragoi G et al. 1999). In the MS-DBB network, it has been shown that despite not being involved in social behavior, it functions to facilitate synchrony of hippocampal neural activity via GABAergic and cholinergic projections (Numan R (ed), 2000). The combination of its direct and indirect anatomical connections with the lateral septum via the SUM (Risold PY, 2004; Atoji Y et al., 2004) allows for an integrated modulation to influence the social behavioral response of the animal as it navigates through its surroundings. With respect to the presence of reciprocal projection, it has been shown that CA2 has got reciprocal projections with the SUM, albeit differences in the spatial distribution of labeled axonal fibers that are dependent on the spread of the injected site(s) in CA2 (Cui Z et al., 2013). This suggests potential differential functional properties to be investigated for such topographical organization in this reciprocal projection between CA2 and SUM.

Taking into account the intrinsic anatomical and physiological properties of CA2, and the complex network of connections with intra- and extra-hippocampal brain structures, it definitely warrants CA2 to be both a control and transmission center that is complete with associated brain regions regulating emotional components (Henke PG, 1990; Moser MB et al., 1998; Meira T et al., 2018). Since CA2 has a perpetual tight clamp on behavioral responses by default, it suggests that only when a stimulus is dissimilar enough against threshold, and whose emotional salience is high enough to warrant attention and action, will CA2 then "release its inhibitory hold" for the respective neuronal pathways to activate and execute downstream effects.

1.2.4 Social memory in CA2

It was approximately 15 years ago when the field of social memory research begin to reveal compelling evidence on the role of hippocampal CA2 in rodent social memory and recognition. A seemingly serendipitous discovery of a peak overexpression profile of the receptor, AVPR1b, in dCA2 (Young WS et al., 2006) sparked curious interest to uncover and understand the functional purpose of a tiny region in the hippocampus with distinct molecular characteristics. Excitotoxic NMDA lesions of the CA2 region in mice showed impaired social memory in lesioned mice when tasked to discriminate between familiar and novel social stimulus in a three-chamber arena as compared to control groups. This impairment persisted even as the task changed to a repeated-exposure setup where the same familiar mouse was continuously re-exposed in the training trials until a novel mouse was presented in the last trial (Stevenson EL et al., 2014).

In a different study, tetanus neurotoxin (TeNT) was injected into the dCA2 region of Amigo-Cre mice that inactivates CA2 pyramidal neuron transmission in a spatial-specific manner (Hitti F et al., 2014). These TeNT mice were then tested for social recognition memory by deploying multiple task schemes, including the familiar-novel 3-chamber discrimination test, re-exposure of the familiar mouse or presenting a novel mouse after an inter-trial interval, and the repeated-exposure setup. All three types of discrimination setups revealed that, in contrast to control mice, the TeNT mice had spent equal bouts of investigation duration on both the familiar and novel stimulus – proof of their impaired social memory. Given that CA2 is the only hippocampal region to receive vasopressinergic inputs from the PVN, evidence from the optogenetic excitation of vasopressinergic terminals in CA2 enhancing social memory lends strength, in addition to the aforementioned experiments, to the theory that CA2 area is involved in social recognition memory (Smith A et al., 2016).

Over time, multitudes of work have gone to show that various hippocampal (Stevenson EL et al., 2014; Hitti F et al., 2014; Smith A et al., 2016; Okuyama T et al., 2016) as well as extra-hippocampal regions, such as the main and accessory olfactory bulbs, medial amygdala etc. (Rossier J et al., 2003; Moyaho A et al., 2015; Gunaydin LA et al., 2014; Richter K et al.,2005; Noack J et al., 2010) are, in one way or another, implicated in different social behaviors. So why the abrupt interest in CA2 specifically for social memory?

Beginning with the characteristics of CA2, visualizations of the region shows that it shares a highly similar pyramidal neuron morphology with that in CA3 (Lorente de No R, 1934; Ishizuka N et al., 1995) in which the neurons in CA2 have similar firing properties to CA3 (Lee H et al., 2015; Lu L et al., 2015). CA2 neurons also has place fields just like those in the other CA regions; and it shares similar synaptic properties like AMPA and NMDA receptor mediated responses with those of CA1 SR pyramidal neurons (Zhao M et al., 2007). Since it shares high degrees of similarity with other CA regions in the hippocampus, it leads one to postulate that, theoretically speaking, the traditional trisynaptic circuit – comprising of a CA3 region that could auto-associate (Grossberg S,1971; Lisman J, 1999; McClelland JL et al., 1985; McNaughton BL et al., 1987; Rolls E, 2010) and execute pattern separation (Colgin LL et al. 2008; McClelland JL et al., 1996; O'Reilly RC et al., 2001) and completion (Colgin LL et al. 2008; McClelland JL et al., 1996; McNaughton BL et al., 1987), and then directly synapsing onto CA1 neurons for information transmission via the Schaffer collateral pathway (Lee I et al., 2005) – could very well detour around CA2 altogether; and rendering CA2 a redundant piece in the hippocampal structure. Yet, the trisynaptic circuit fails when it was put to the test for social recognition with CA2 silenced (Stevenson EL et al., 2014; Hitti F et al., 2014). The fact that other non-social components of behavior and memory,

such as simple locomotor activity, hippocampal-dependent spatial and contextual or auditory memory (Hitti F et al., 2014) and generic olfactory processing (Stevenson EL et al., 2014), remained intact while CA2 is silenced, proves that this hippocampal region plays an indispensable role for the social component of hippocampal-dependent episodic memory.

On top of a conflict in ascribing a functional role to CA2 – whether it is implicated only during the encoding phase of social memory (Smith A et al., 2016), or in both encoding and recall of social memory (Hitti F et al., 2014), the functional relationship between CA2 and vCA1, another hippocampal region shown to be the storage site of social memory engrams for recall (Okuyama T et al., 2016), remains to be demonstrated. Furthermore, social memories formed from epochs of social interactions are not necessarily a one-to-one occurrence. For instance, one may come into contact with multiple social stimuli successively at different time points or interact with multiple stimuli simultaneously (e.g. at a conference, or in a meeting room). As of present, such contexts that mimic the natural world were not taken into consideration when designing a behavioral paradigm for probing social memory. Therefore, our current study will show results from investigations conducted to address the functional significance of CA2 and its functional relationship to vCA1 using a behavioral paradigm designed with a relatively closer mimicry of social interactions in natural world.

1.3 Oxytocin and Oxytocin Receptors

This section will briefly introduce the neuropeptide, oxytocin (OXT) and its associated receptor, OXTR; including their neuromodulatory effects and the regions upon which they act to exert an effect. Due to the fact that it is beyond the scope in this thesis to give an in-depth, breadth-wide coverage on OXT and OXTR in its entirety, the literature mentioned will be directed towards their modulatory effects pertaining to social recognition. Also, some mention of AVP, a closely related neuropeptide to OXT, will be made throughout this section as a means of comparative discussion.

OXT and AVP are important chemical signals acting in both the peripheral, to regulate physiological functions, and central systems, to regulate different types of social and affiliative behaviors. The two neuropeptides are closely related nonapeptides differing at only their 3rd and 8th amino acid positions (Gruber CW, 2013). Depending on their site of release, both OXT and AVP can take on different roles as neurotransmitters or neuromodulators; which in turn is dependent on the mode of transmission – autocrine, paracrine, or endocrine – at various tissue regions at which they take effect.

1.3.1 Oxytocin and its transmission

Both neuropeptides are preserved and duplicated through evolution: [Lys^{8}]conopressin in mollusk Lymnaea stagsnails guides male copulatory behavior (van Kesteren et al., 1995); arginine vasotocin in male teleost fish and isotocin in females influences grunting during reproductive behavior (Goodson JL et al., 2000a; Goodson JL et al., 2000b); mesotocin receptor distribution in the lateral septum correlates with flock size in some bird species wherein mesotocin administration and the associated antagonist correlates with the increase or decrease in flock size (Goodson JL et al., 2009). In the mouse, rat and human genome, OXT and AVP genes are located on the same chromosome separated by 3.5-12 kbp intergenic region despite being in opposite transcriptional orientations (Hara Y et al., 1990; Mohr E, S .E., Richter D et al., 1988; Mohr E, B.U., et al., 1988; Sausville E et al., 1985; Schmitz E et al., 1991). In the brain, OXT is synthesized by two separate neuronal populations: magnocellular and parvocellular cell populations (Krieg WJS, 1932) in the PVN and the supraoptic nuclei (SON) (Farina Lipari E, Valentino B, 1993; Farina Lipari E et al., 1995). Despite some controversy in cell type classification based on their morphology and efferent projections due to technical limitations (Dolen G et al., 2013; Knobloch HS et al., 2012; Ross HE et al., 2009; Swanson LW et al., 1980), current observations that the PVN contains both magnocellular and parvocellular OXT neurons (Castel M et al.,1988; van den Pol AN, 1982; Castel M et al., 1997) while the SON contains only magnocellular OXT neurons (Castel M et al.,1988), and the known differences in their relative spatial and axonal projection distributions give rise to the ideation that the type of OXT peptide transmission (i.e. synaptic, paracrine, endocrine) depends on its type of cellular source. Therefore, the downstream consequences triggered by OXT will be dependent on the targeted sites at which these projection fibers affect.

Magnocellular neurons release OXT from large dense core vesicles (LDCVs) that are located in large numbers at somatic, dendritic and axon terminals release sites, while parvocellular neurons release OXT from medium-sized vesicles in comparatively smaller numbers only at the axon terminals (van den Pol AN, 2012). Magnocellular LDCVs release OXT via endocrine transmission from axonal terminals, where the peptide is released directly into the bloodstream for peripheral circulation (van den Pol AN, 2012). They also release OXT via paracrine transmission from the dendrites and cell bodies of magnocellular neurons (de Kock CPJ et al., 2003), where OXT is released directly into the cerebrospinal fluid (CSF) for central circulation (Ludwig M et al., 2006; Castel M et al., 1997; Pow DV et al., 1989; Landry M et al., 2003; Simmons ML et al., 1995). Comparing endocrine and paracrine modes of OXT transmission where OXT functions as neuromodulators, the former is faster in its diffusion but has a significantly shorter half-life in the blood as compared to that for the latter in the CSF (Ludwig M et al., 2006; Mens WB et al.,

1983). In contrast, the release of OXT via synaptic transmission at axonal terminals are proposed to be from parvocellular neurons (Dolen G et al., 2013), where OXT functions as neurotransmitters, is discharged rapidly and in much smaller controlled quantities that are restricted to the synaptic space contacted by the axon terminal (van den Pol AN, 2012).

Since OXT modulates numerous regions in the body, understanding the various modes of transmission for the OXT peptide will present crucial inferences for insights as to how it possibly affects different target sites to regulate biological processes such as ovulation, parturition, lactation, sexual behavior, social interaction and social memory (Legros J, 2001; Raam T et al., 2017; Oettl LL et al., 2016). Another key factor is the expression profiles of the OXTR. In regions where both OXTR and AVPR are found to be expressed (e.g. OXTR and AVPR1b in Hippocampal CA2 region (Smith A et al., 2016; Tirko NN et al., 2018) will have to account for the selective binding affinities of both neuropeptides to their respective receptors types, the types of neurons they are respectively expressed in, and the possibility of cross-talk between OXT and AVP given the similar chemical structures of their associated receptors (Young LJ, F.-C.L, 2012; Song Z et al., 2018).

1.3.2 Oxytocin Receptor and its distribution

OXT only has one known receptor – the OXTR, a class I G-protein coupled receptor (Hoyle C, 1999; Gimpl G et al., 2001) that is coupled to the Gq/11a class GTP binding proteins which activates phospholipase C pathway (AVP has three: V1aR, V1bR, V2R (Caldwell HK et al., 2006; Barberis C et al., 1996; Hasunuma I et al., 2013). Even though the binding of OXT is only ten-fold more selective for the OXTR over receptors for arginine vasopressin, its receptor OXTR has a 100-fold higher affinity for OXT over AVP. AVP, on the contrary, exhibits equal binding affinity to all 4 types of receptors mentioned (Chini B et al., 1995; Hawtin SR et al., 2000; Kimura T et al., 1994; Lolait SJ et al., 1995; Thibonnier M et al., 2001; Song Z et al., 2014).

OXTR are extensively expressed throughout the brain; and it is found that its expression profiles are distinctively high in the hippocampus, amygdala, striatum, suprachiasmatic nucleus, bed nucleus of stria terminalis, piriform cortex, auditory cortex, and the brainstem (Buijs RM and Swaab DF, 1979; Sofroniew MV, 1983; Young WS 3rd and Gainer H, 2003; Meyer-Lindenberg A et al., 2011; Grinevich V et al., 2016; Mitre M et al., 2016). Interestingly, OXTRs have a highly restricted pattern of distribution in the hippocampus; especially found to be expressed in primarily the pyramidal neurons within the CA1,

CA2 and CA3 areas (Yoshida M et al., 2009, Lin YT et al., 2018, Raam T et al., 2017), as well as in GABAergic inhibitory neuron in CA1 (Owen SF et al., 2013).

1.3.3 Oxytocin, Oxytocin Receptors, and their roles in social memory

In the field of social memory, several works focusing on different brain regions show that the OXT peptide and OXTR are crucial players in influencing the state of social memory seen from observable behavior in rodent models. The following will share and discuss what can be concluded or inferred with regards to a few regions of the brain where OXT-OXTR could affect social recognition memory, i.e. the ability to discriminate the familiar from the novel.

Examples of studies that targets specific brain regions include those that showed the implication of the amygdala as one of the potential sites for social memory (Ferguson JN et al., 2001; Choleris E et al., 2007). Using OXT and OXTR knockout mice, the impaired social memory was observed from the increased duration spent on investigating a previously familiarized mouse (Takayanagi Y et al., 2005; Ferguson JN et al., 2001); and that this impairment was rescued by intracerebroventricular infusion of OXT into the amygdala of OXT knockout mice (Ferguson JN et al., 2001). Using genetic manipulations, knockdown of the OXTR gene in the amygdala have resulted in social recognition impairments from the observation of decreased investigation times for the novel (Lee HJ et al., 2008; Choleris E et al., 2007) and familiar mouse. It is also shown that knocking down the OXTR gene resulted in a general decline in sociability (i.e. the tendency to investigate a social stimulus) in the three-chamber discrimination task (Pobbe RLH et al., 2012). However, the tests of social recognition memory and sociability targeting the amygdala are mostly conducted without an inter-exposure interval; hence resulting in a controversial conclusion that such investigations are considered to be founded on basis of social novelty detection (Crawley JN et al., 2007). Additionally, other studies have otherwise shown that the major function of the amygdala is directly implicated in attenuating fear response in fear conditioning experiments (Huber D et al., 2005; Viviani D et al., 2011). Taken together, it implies that the action of OXT in the amygdala is suggested to be likely for the modulation of social anxiety rather than for enhancing social memory itself.

Another brain region of interest where OXT-OXTR coupling can modulate social-related memory is the olfactory system. An early study of OXT infused into the olfactory bulb (OB) of male rats showed that it facilitates social recognition with either a 30-minute and 120-minute interval compared to the control

group, but application of an OXT antagonist into the OB failed to impede social recognition at either time intervals (Dluzen DE et al., 1998a). This probably provides a first hint that OXT likely functions to modulate social recognition via odor processing regions/pathways as the encoded social olfactory information may be long transmitted to higher order brain regions for further processing and storage. A more recent study investigated the role of OXT released from the PVN of female rats using optogenetics to excite the oxytocinergic neuronal cell bodies in the PVN, and also investigated the role of the densely OXTR-expressing neurons in the anterior olfactory nucleus (AON) using conditional deletion of OXTR (Oettl LL et al., 2016). The former experiment is able to demonstrate that OXT release from the PVN is able to prolong the retention of social recognition from 30 minutes to two hours, and that the latter showed that the deletion of OXTR in the AON affected only olfaction-dependent sampling behavior and consequent conspecific recognition while leaving non-social odor discrimination unaffected. The authors acknowledged that their experiments, at best, suggest that an OXT-enhanced state is able to boost stimulus selectivity and information extraction of social cues, and that a stronger OXTR activation in the AON is speculated to reduce background firing noise while amplifying social odor responses. Taken together, these investigations can, at the very least, provide basis for the functional roles of PVN OXT and OXTR expressed in AON as that of the extraction and signal-to-noise ratio amplification of social information, which indirectly influences the behavioral outcome of social recognition.

The OXTR expression profile is distributed heterogeneously in the central nervous system, including in the hippocampus (de Kloet ER et al., 1985; van Leeuwen FW et al., 1985); and it, too, is investigated as one of the regions wherein the OXT peptide affects. Numerous interventive experiments such as intracerebroventricular administration of OXT antagonists have shown to inhibit social recognition (Engelmann M et al., 1998); and likewise for genetic manipulations such as OXT and OXTR knockout studies in rodents, where they show that knockout mutants only suffer from an impaired ability to discriminate between social stimulus, but are spared from deficits in general sociability (Ferguson JN et al., 2000; Takayanagi Y et al., 2005; Crawley JN et al., 2007; Ragnauth AK et al., 2005; Lee YS et al., 2009; Winslow JT et al., 2000).

Taking all of these studies into account, it is no doubt that all of them have detailed reports on the indispensable nature of OXT for regulating social behaviors; and that the lack of OXT-OXTR regulation

commonly results in elevated levels of aggression (Winslow JT et al., 2000; Ragnauth AK et al., 2000). However, the regulatory effects of OXT-OXTR coupling is not necessarily restricted to experiments involving social stimulus and cues. Though conditional deletion of OXTR from forebrain excitatory neurons, including the hippocampal pyramidal neurons, indicated that OXTR are necessary for refined levels of discrimination such as intrastrain social recognition (Macbeth AH et al., 2009), they are also implicated in the reduction of freezing behavior during acquisition, as well as during context and cue retention (Pagani JH et al., 2011). Hence, there is nothing quite "social" about OXT or OXTR *per se*; and it makes it harder to disambiguate the multiple regulatory roles of OXT-OXTR to identify specific mechanisms that underlie recognition in the aspect of social memory.

Fortunately, recent advancements made the use of a floxed OXTR mouse line possible for a more spatially precise manipulation of the OXTR in the hippocampus (Lee HJ et al., 2008) – a tissue structure widely known for both the encoding and retrieval of episodic memories; and also a region of OXTR expression. It can now be, for example, demonstrated that targeted ablation of OXTR using viral Cre recombinase injected into OXTR-expressing regions such as the hilar region of the dentate gyrus (DG), dCA2 and CA3a in floxed OXTR mice resulted only in reduced social discrimination while keeping sociability and non-social hippocampal-dependent memory – novel object recognition – unaffected (Raam T et al., 2017). On top of using a repeated-exposure social discrimination paradigm, another similar study which also targeted CA2/CA3a neuronal regions for OXTR ablation, used a three-chamber discrimination paradigm that includes a 7-day interval to show such manipulation impaired long-term social memory without compromising anxiety-like behavior in the open field, elevated plus maze, and novelty suppressed feeding tests (Lin YT et al., 2018).

Although results from the aforementioned pivotal studies are confounded by manipulations that might have spillover effects to implicate areas like DG and CA3a in the data collected as well, they do prove that the activation of OXTR-signaling pathways in the hippocampus are crucial for regulating a functional social recognition memory; which are consistent with findings that implicate the OXTR-expressing hippocampal CA2 region in social behavior (Wersinger SR et al., 2002; Young WS et al., 2006; Hitti F et al., 2014; Pagani JH et al., 2014; Stevenson EL et al., 2014; Smith A et al., 2016).

Taking things one step further in depth, studies investigating the mechanism of OXT-OXTR signaling now attempt to rationalize the physiological and, perhaps, behavioral, phenomena behind manipulations of the OXT-OXTR pair in the hippocampus. OXTR inactivation studies revealed the involvement of both excitatory and inhibitory neurons in the hippocampus (Tirko NN et al., 2018; Owen SF et al., 2013). By

uncovering a direct anatomical projection for OXT release into CA2 from the PVN, and then activating those oxytocinergic projections into CA2 for electrophysiological recordings, it was illustrated that the regulation of the resultant OXTR signaling pathway caused repetitive firing in CA2 arising from various signaling events, ionic mechanisms, and concerted circuit actions (Tirko NN et al., 2018). Of note, it was found that both CA1 pyramidal neurons and parvalbumin (PV) -positive interneurons are directly and independently responsive to the applied OXTR-selective agonist, TGOT (Owen SF et al., 2013). This implies that the overarching function of the OXT analog-driven OXTR signaling serves to enhance the excitability of CA2 pyramidal cells, which drives a bursting pattern output projected onto CA1 neurons to favor excitation over inhibition, while the input from GABAergic inhibitory neurons facilitated that effect by increasing the intensity of the TGOT-induced bursts. Although these findings do provide a convincing theory on the molecular mechanism between the two types of behavioral observation (i.e. the loss of social recognition function in CA2/CA3a-specific OXTR knockout mice (Raam T et al., 2017; Lin YT et al., 2018) and that of CA2-silenced mice (Hitti F et al., 2014), the actual social behavioral effects posited by the modulation of CA2 output to vCA1 due to OXTR signaling remains to be demonstrated. Additionally, it is worth of mention that while the findings of such an amplified information transfer from dCA2 to CA1 facilitated by OXTR signaling claimed to be reconciling genetic and anatomical literature specific to social recognition memory, its results are based on recordings done in dorsal CA1 (dCA1) not ventral CA1 (vCA1). Therefore, it contradicts with previously shown evidence of social memory being stored in vCA1, and not dCA1 (Okuyama T et al., 2016). As such, it remains to be confirmed if the theory of an amplified, fine-tuned dCA2 output to vCA1 – the storage site of social recognition memory – can be rationalized with said mechanism.

Chapter 2. Hippocampal microcircuits for social memory specification

2.1 Introduction

For social animals including humans, it is crucial for their survival to not only distinguish the familiar from novel conspecifics but also between multiple previously familiarized individuals (Tibbetts EA et al., 2007), as in most scenarios in the natural world. The former ability is broadly referred to as "social recognition" while we here term the latter as "social specification". Studies on human patients with hippocampal lesions have demonstrated the indispensable role of the hippocampus in social recognition (Smith et al., 2014). Notable is the case of social specification memory in the human hippocampal neurons that respond to a specific familiar individual, popularly referred to as the "Jennifer Aniston" cells (Quiroga, Reddy, Kreiman, Koch, & Fried, 2005). Similarly, an animal's ability to recognize the hierarchical social dominance in its group suggests that it too can distinguish between multiple familiar individuals (Wang et al., 2011).

Recent studies on mice have revealed the indispensable role of the hippocampal subregion dorsal CA2 (dCA2) in the social recognition memory (Hitti & Siegelbaum, 2014). Furthermore, social engram neurons for a specific social recognition memory have been identified in the downstream ventral CA1 (vCA1) (Okuyama, Kitamura, Roy, Itohara, & Tonegawa, 2016). It is also known that dCA2 has intrinsic connections with ventral hippocampus along the longitudinal axis of the hippocampus (Tamamaki N et al., 1988) which is crucial for the social recognition memory (Meira T et al., 2018; Raam T et al., 2017). Thus, both dCA2 and vCA1, along with their intra- and extrahippocampal circuits serve to facilitate the social memory of the recognition type.

However, the exact anatomical site for storing and retrieving a more precise type of social memory – the social specification memory – and its underlying mechanisms remains undiscovered. This study thus aims to determine whether the hippocampal sub-regions implicated in the recognition memory also store information for social specification memory; and if so, to identify the underlying physiological and circuit element mechanisms for this phenomenon. Here, we show that vCA1 is the site of the storage for social specification memory; and present the finding of multiple feedforward inhibition motifs within the dCA2-vCA1 microcircuit that are crucial to progressively refine the specificity of social memory.

2.2 Results

Social recognition memory can be quantified by measuring the duration of interaction between familiar and novel mice (social discrimination test). This test capitalizes on the innate behavior of rodents to spend more time interacting with a novel individual compared with a familiar one. Neural substrates for the storage and retrieval of memories are referred to as engrams (Josselyn SA and Tonegawa S, 2020; Tonegawa S et al., 2015). Our previous study carried out using the memory engram identification method (Liu X et al., 2012; Ramirez S et al., 2013) showed social memory is formed and stored in the vCA1 subfield of mouse hippocampus (Okuyama T et al., 2016). This memory could be retrieved by natural cues – a re-exposure to the familiar mouse – only for up to a half hour after formation (Okuyama T et al., 2016). However, labeling of the engram cells with channelrhodopsin (ChR2) and their subsequent artificial activation by laser light demonstrated that the memory is actually maintained for at least one day despite being inaccessible to natural recall cues in a half hour (Okuyama T et al., 2016) as the memory turns to a "silent" state (Roy DS et al., 2017; Ryan TJ et al., 2015). We took advantage of these properties of social memory to investigate whether the vCA1 memory can be specific for an individual familiar mouse (i.e. social specification memory).

Two groups of wild-type mice, group-I and group-II, received a bilateral injection of a mixture of AAV9-cfos:tTA and AAV9-TRE:ChR2-EYFP in the vCA1 (Figure 1A) and were kept on doxycycline (Dox) diet. Both groups of mice were familiarized with two sets of mice, mouse-A and mouse-B, separately and successively (Figure 1B). For group-I mice, the labeling window of the engram cells with ChR2-EYFP was open during mouse-A familiarization period by removal of Dox from the diet, whereas the window was kept closed during mouse-B familiarization period. The reverse is true for group-II mice, where the labeling window was closed during mouse-A familiarization period and was open during mouse-B familiarization period. Optogenetic reactivation in a three-chamber social discrimination test (SDT) were conducted 72 hours after each familiarization period, by which time the engrams for the familiar mice would be in a silent state, between a familiar mouse (mouse-A or mouse-B) and a novel mouse (mouse-N). This paradigm is, henceforth, referred as the "social specification behavior paradigm". For both group-I and group-II mice, optogenetic reactivation of ChR2-labeled engram neurons in vCA1 resulted in behavioral recall of the corresponding familiar mice (Figure 1D, dark blue bars) but not for the other familiar mice wherein the engram was not labeled (Figure 1D, light blue bars). These results suggest that vCA1 is capable of storing social memory with specificity for each of the individual familiar mice.

Next, we similarly labeled dCA2 neurons with ChR2 that were active during social interactions. Although dCA2 neuronal activity is crucial for social memory (Hitti F et al., 2014), dCA2 neurons do not intrinsically express c-Fos upon learning (Supplementary figure. S1, A – F). Hence, we used the engineered synthetic promoter of an alternative immediate early gene Arc, an enhanced synaptic activity-responsive element (eSARE) (Kawashima T et al., 2013), to label dCA2 neurons with ChR2 that were activated by social interaction(Figure 1, E – G, Supplementary figure S1, J – O). Using the same social specification behavior paradigm, wild-type mice were familiarized with both mouse-A and mouse-B, with either mouse-A neurons or mouse-B neurons labeled with ChR2 in separate experimental batches. However, unlike the effects observed by reactivating vCA1 neurons, optogenetic reactivation of the labeled dCA2 neuronal population had no effect on social memory recall (Fig. 1H); therefore suggesting that dCA2 store neither social recognition memory (Okuyama T et al., 2016) nor social specification memory.

To verify that the dCA2 neuronal activity induced by social interaction nevertheless represents social information, AAV9-hsyn:DIO-eArchT-EYFP was injected into dCA2 of Map3k15-Cre transgenic mouse line (Kohara K et al., 2014), in which Cre is expressed specifically in CA2 pyramidal neurons (Figure 1I; Supplementary figure 2, A – B). Optogenetic inhibition of dCA2 activity specifically during memory encoding, but not during recall, resulted in social memory impairment (Supplementary figure 2, C – E), suggesting that dCA2 contributes to social memory formation during encoding. Therefore, we hypothesized that dCA2 transfers social information to vCA1 to form social memory engrams in the latter. To test the hypothesis of this functional relationship between dCA2 and vCA1, wed silence dCA2 neuronal firing during social memory formation by expressing inhibitory DREADD receptors (hM4Di) in dCA2 neurons. AAV9-hsyn:DIO-hM4Di was injected into dCA2 of Map3k15-Cre mice, and an intraperitoneally injected hM4Di ligand, clozapine-N-oxide (CNO), 45 min before the encoding of social memory for the labeled mouse stimulus, was administered (Fig. 1I to N). Similar to the previous experiments (Figure 1, A – D), the mixture of AAV9-cfos:tTA and AAV9-TRE:ChR2-EYFP was injected into vCA1 to label vCA1 social memory engram neurons with ChR2. It was shown that the chemogenetic inhibition of dCA2 activity during the encoding phase of the respective activity-dependent labeling epoch failed to elicit social memory retrieval despite optogenetic reactivation of the vCA1 neurons (Figure 1, O to R). This suggests that in mice, social information is conveyed from dCA2 to vCA1 for the formation of mouse-A engram cells in vCA1 and subsequent recall of the memory. Control experiments conducted without CNO injection (i.e. saline injection group) or without DREADD receptor expression (i.e. EYFP expression group) did not show such impairments in social memory engram in vCA1 (Supplementary

figure S3, A – C). Additionally, another control group without light stimulation allowed us to confirm that social memory retrieval was attributed specifically to the optogenetic reactivation of vCA1 social memory engram, and not a residual effect of simply expressing ChR2 (Supplementary figure S3D).

We next investigate the anatomical connection from dCA2 to vCA1 by injecting AAV9-EF1α:DIO-ChR2-EYFP into the dCA2 of Map3k-15 Cre mice to label CA2 axonal fibers innervating the dorso-ventral (DV) axis of the hippocampus (Supplementary figure S4, A – B). We found that the density of EYFP-labeled fluorescent axons decreased drastically along the DV axis. dCA2 injections yielded prominent fiber density in iCA2 and dCA1, but not further downstream ventrally in vCA2 (Supplementary figure S4C – intensity profile for dCA2 injected mice are currently under construction; D: temporary inclusion for illustration; not part of actual figure). Similarly, iCA2 neurons directly project to vCA2 and iCA1, but not vCA1.

To verify the synaptic connections from dCA2 to vCA1, ChR2-EYFP expressing fibers from dCA2 were optogenetically stimulated with blue light and downstream pyramidal neurons in iCA2 were patched (Figure 2, A – C). Out of a total 31 iCA2 neurons, 20 cells were patched in current clamp and 11 cells in voltage clamp. By varying the holding potential of a current clamped neuron, we observed changes in the dynamics of the neuronal response to 10 Hz blue light stimulation (Figure 2D, G). 11 cells showed exclusively excitatory responses to stimulation of dCA2 fibers, and 20 cells showed EPSPs at resting potential and predominant IPSPs when depolarized to -55mV (E+I). We observed a 100% connection probability between dCA2 and iCA2. Out of these 20 cells, the 5/20 (25.0 %) in current clamp showed a slow GABA-B inhibition, whereas 7/20 (35.0 %) showed predominantly GABA-A mediated inhibition (Figure 2D).

We also observed a feed-forward circuit motif in the projection blocks of iCA2-vCA2 and vCA2-vCA1. To verify each step of the dorso-ventral microcircuit, Cre-dependent ChR2-EYFP virus was injected into iCA2 to patch vCA2 neurons and vCA2 to patch vCA1 neurons in Map3k15-Cre mice, respectively. We patched and recorded post-synaptic responses in a total of 16 vCA2 pyramidal neurons (13 current clamp and 3 voltage clamp), and 36 vCA1 neurons (20 current clamp and 16 voltage clamp) (Figure 2, E – G). In the iCA2-vCA2 connection, we observed exclusively excitatory responses in 5/16, E+I responses (GABA-A mediated inhibition) in 3/16 when depolarized to -55mV and no responses in 8/16 neurons (Figure 2H). Similarly, in the vCA2-vCA1 connection, 7/20 vCA1 cells showed exclusively excitatory

responses, and 11/20 cells showed a combined E+I response (GABA-A mediated inhibition) when depolarized. As in the case for dCA2-iCA2, the connection probability between vCA2 and vCA1 was 100%. No slow GABA-B components were observed in either iCA2-vCA2 or vCA2-vCA1. Furthermore, when tested under voltage clamp, E+I components showed a complete loss of the GABA-A inhibitory current upon the application of Gabazine, and a loss of the GABA-B component upon application of CGP55845 (Figure 2H). Additionally, consistent to our anatomical data (Supplementary figure S4), we found no evidence of functional connections from dCA2 to vCA1 (Figure 2I, 20 cells).

To further investigate the functional significance of repeated feed-forward inhibition motifs in social memory, we labeled neuronal populations which encode the representations of familiar mouse-A and familiar mouse-B, respectively. An AAV9-eSARE:tTA and AAV9-TRE:Fluorescent Timer (FT)-Slow mixture was separately injected into dCA2, iCA2, and vCA2 of Map3k15-Cre mice. In addition, AAV5-EF1a:DIO-H2BGFP (nuclear localized EGFP) was co-injected to visualize the somata of CA2 neurons (Figure 3A). Since the different fluorescence proteins of FT-Slow have different peak expressions and decay curves, any reactivated neuronal population will naturally change in color, from blue to red, over course of 12 to 72 hours after injection (Okuyama T et al., 2016; Subach FV et al., 2009). The neural population activated by first and second social interactions which took place 3 days apart could therefore be distinguished by the red and blue colors respectively (Figure 3, B – J). The proportions of cells reactivated by interactions with the re-exposure of familiar mouse-A are found to be equally high (50 - 60%) in dCA2, iCA2, and vCA2. However, this reactivation ratio, obtained from the successive exposure to mouse-A and then mouse-B, progressively decreased as the neural processing proceeded through the dCA2-iCA2-vCA2 circuit (Figure 3, K – L). This suggests that the ability for the eventual discrimination between multiple familiar mice increases at the level of engram neurons as they become gradually distinct along the CA2 DV projections in a “relay-like” manner.

Feed-forward inhibition has been reported in phenomena such as the tuning of visual perception and the sharpening of CA1 place field (Hu H et al., 2014). To examine whether the repeated feed-forward inhibition motifs in CA2 (Figure 2) contributes to social memory specification, we chemogenetically silenced GABAergic neurons in the early relay stations – dCA2 and iCA2 – and assessed its effect on social specification memory at the engram neuron population level as well as the behavioral level. We expressed inhibitory DREDD receptor, hM4Di in GABAergic neurons in dCA2 and iCA2 by injecting AAV9-hsyn:DIO-hM4Di bilaterally into dCA2 and iCA2 of Slc32a1-Cre mice in which Cre was expressed only in GABAergic neurons (Figure 3M), and CNO was injected 45 min before social memory

encoding to inhibit GABAergic neuron activity (Figure 3N). We investigated for any effect on the specification at the neuronal population level similar to that done for Figure 3K, and found that the proportion of reactivated cells in vCA2 visualized after successive epochs of social interaction with familiar mouse-A and then familiar mouse-B was significantly greater in the Slc32a1-Cre mice compared to wild-type mice (Figure 3O). Furthermore, social specification memory was found to be impaired in Slc32-Cre mice compared to wild-type mice when GABAergic inhibitory effects was disrupted (Figure 3, P – S). These data suggest that engrams for familiar mouse-A and familiar mouse-B became less distinguishable; thus resulting in non-specific reactivations that compromised social specification memory without the fine-tuning contributed by feed-forward inhibition in CA2 microcircuits.

Finally, we investigated whether modulation from OXTR-signaling processes within the dCA2-vCA1 microcircuits contributes to the social recognition and social specification memories. We focused on investigating the functional role of OXTR expressed in the excitatory neurons. It has recently been reported that OXTR is expressed in dCA2 excitatory neurons (~ 96 % of RGS14 expressing neurons) (Tirko NN et al., 2018) and that this expression is crucial for the modulation of social recognition memory (Raam T et al., 2017). Similarly, we found that OXTR was expressed in the entire CA2 fields, including iCA2 and vCA2, as well as in vCA1 pyramidal neurons (but comparatively lesser in dCA1, consistent with (Tirko NN et al., 2018). We, too, found that a large proportion of the OXTR-expressing neurons are excitatory (Figure 4, A – E). To eliminate OXTR expression in neurons of targeted regions, $OXTR^{flox/flox}$ mice were crossbred with Trpc4-Cre (where Cre is expressed in both vCA1 and dCA1 pyramidal neurons) and Wfs1-Cre (Cre-specific for dCA1 pyramidal neurons), Map3k15-Cre (Cre-specific for CA2 pyramidal neurons), and Vgat-Cre (Cre-specific for GABAergic neurons). Trpc4-Cre/$OXTR^{flox/flox}$ and Map3k15-Cre/$OXTR^{flox/flox}$ mice displayed impaired social discriminatory behavior whereas Wfs1-Cre/$OXTR^{flox/flox}$ and Vgat-Cre/$OXTR^{flox/flox}$ did not (Figure 4,F – I). This demonstrates the pivotal role of OXTR expression in modulating vCA1 and CA2 excitatory neuronal activity to promote social recognition memory. Additionally, AAV2-EF1a:Cre or AAV5-CaMKII:Cre injection into vCA1 $OXTR^{flox/flox}$ augmented the behavioral impairment observed in Figure 4, F – I during SDT (Figure 4, J – K).

Next, we examined whether the expression of OXTR in excitatory neurons in the hippocampal social microcircuits (i.e. dCA2 → iCA2 → vCA2 → vCA1) facilitates social specification. We found that OXTR ablation in dCA2 and iCA2 resulted in a significant increase in the proportion of reactivated neurons in vCA2 using our social specification behavior paradigm with successive exposures to mouse-A and mouse-B (Figure 4, L – N). Consequently, this high rate of reactivation impaired the formation of

social specification memory at the behavioral level (Figure 4, O – S). These results, taken together with the data shown in Figure 3, M – S, suggest that both the appropriate modulations of excitatory and inhibitory neurons, contributed respectively from OXTR-signaling effects and the feed-forward inhibition "relay" from CA2 to vCA1, are crucial for the formation and retrieval of both social recognition and specification memories.

2.3 Conclusion and Discussion

2.3.1 Conclusion

In this chapter, we have demonstrated three main conceptual findings for the hippocampal dCA2-vCA1 microcircuit: 1) a distinct functional significance for CA2 and vCA1; wherein CA2 functions to encode and fine-tune social specification memory as neural signals traverse from dCA2 to iCA2 to vCA2, while vCA1 functions mainly as the storage site of the encoded social memory for eventual retrieval; 2) a functional dependency of vCA1 on CA2 for storing the respective population-based neuronal codes for the effective recall of individual social stimulus; and 3) a modulatory mechanism via synergistic effects of a GABAergic feedforward inhibitory network and OXTR signaling for fine-tuning progressively specific neuronal populations for multiple unique social stimuli encoded.

Via a novel social specification behavior paradigm, we show that distinct neuronal populations exist for individual social stimulus (i.e. social memory engrams). Their reactivation resulted in the recall of only the corresponding labeled familiar stimulus despite having interacted and familiarized with multiple stimuli – a phenomenon we termed as "social specification memory". However, this phenomenon of a successful recall is only prominent when reactivating social memory engrams in vCA1, but not in dCA2 (Figure 1, A – H). Such a finding provides preliminary hints of a differential functional significance of vCA1 and dCA2; which was reinforced by loss of function experiments in dCA2 to show that dCA2 is only implicated in the encoding phase of social memory (Supplementary figure S2D). Thereon, it instigated the experiment of probing for the functional relationship between dCA2 and vCA1 which enable animal subjects to recall a familiar social stimulus. Following which, it was proven that vCA1 is dependent on dCA2 for the subsequent recall of a specifically-labeled set of neurons corresponding to a particular social stimulus (Figure 1, I – R).

Since dCA2 has been implicated in social memory, and that vCA1 has been shown to store social memory engrams (Raam T et al., 2001; Stevenson EL et al., 2014; Hitti F et al., 2014; Smith A et al., 2016; Lin YT et al., 2018; Okuyama T et al., 2016), we examined for a direct anatomical projection connecting dCA2 to vCA1 which may explain the elicited behavioral results obtained from Figure 1. However, we found no such direct projection (Figure 2I). Instead, we discovered, with controlled amounts of viral injections into Map3k15-Cre mice, that there exists a feedforward, "relay-like" projection from dCA2 to iCA2, then from iCA2 to vCA2, and finally from vCA2 to vCA1 (Figure 2, D – F). Within each projection segment (i.e. dCA2 → iCA2, iCA2 → vCA2, or vCA2 → vCA1), we found a composition of excitatory (pyramidal) and inhibitory (GABA-A and/or GABA-B interneurons) neurons. This led to the hypothesis that the neuronal population encoding for social information becomes progressively fine-tuned when information is transmitted from the dorsal to ventral sub-regions of CA2 by means of "relay-like" projections; thereby allowing subject mice to effectively recall whichever familiar stimulus it has previously interacted with – conditional on an appropriately labeled social memory engram for that stimulus.

By modifying the aforementioned social behavioral paradigm to include a temporal-specific inhibition of interneuron signaling in early relay sub-regions in CA2, we discovered that the distinct population codes for different social stimuli turned out to be indistinguishable as compared to their wildtype controls (Figure 3, A – O). Inevitably, the corresponding behavioral results provided additional evidence to the notion that disrupting inhibitory regulation in the CA2 network impedes the formation of a distinct population code for each familiar stimulus (Figure 3, M – O). This results in the failure to optogenetically reactivate social specification memory for a particular familiar conspecific (Figure 3, P – T). Hence, it suggests that CA2 interneurons regulate the segregation of population codes for different stimuli in a feedforward, "relay-like" manner as well; and that each sub-region of CA2 is indispensable for this purpose of fine-tuning social population codes.

Apart from the fact that disrupting the inhibitory network resulted in an impaired social specification memory, crippling the regulatory signaling pathway which influences the activity of excitatory neurons has also been shown to impair social specification memory. Our findings, based on a series of complimentary social recognition memory tasks; where we used subject mice crossed from different combinations of transgenic lines to show that the OXTR-signaling pathway is the major contributor to regulating CA2 pyramidal neurons activity for social memory (Figure 4, F – K). This is supported by our *in situ* hybridization results showing dense OXTR expression profiles in dCA2, iCA2, vCA2, and vCA1 (Figure 4, A – E); which is also substantiated by previous work from literature that not only shows similar

OXTR expression profiles in the mentioned CA2 and vCA1 regions, but also the presence of direct oxytocinergic fiber projections from PVN to CA2, and behavioral evidence of impaired social memory with the ablation of OXTR in mice as well (de Kloet ER et al., 1985; van Leeuwen FW et al., 1985; Ferguson JN et al., 2000; Takayanagi Y et al., 2005; Crawley JN et al., 2007; Winslow JT et al., 2000; Ragnauth AK et al., 2005; Lee YS et al., 2009; Ferguson JN et al., 2001; Macbeth AH et al., 2009; Lee HJ et al., 2008; Raam T et al., 2017; Lin YT et al., 2018; Tirko NN et al., 2018). More specifically, with a spatially controlled ablation of OXTR in selective CA2 sub-regions (similar to that performed for Figure 3, M – O), we again observed a high reactivation rate with indistinguishable population codes post-encoding of the different social stimuli (Figure 4, L – N). The attempt to optogenetically reactivate said labeled set of neurons revealed a similar impairment of social specification memory at the behavioral level (Figure 4 O – S; compared with Figure 3, P – T).

In the entirety of this investigation, we showed that for social specification memory, dCA2 and vCA1 exhibited differential functional roles in the encoding and retrieval of stimulus-specific information respectively. In addition, social specification memory requires the concerted effort of transmitting encoded information from dCA2, iCA2, vCA2, and to vCA1 in a “relay-like” manner; in which the information transfer process is synergistically modulated, also in a “relay-like” manner, by feedforward inhibitory activity and OXTR-signaling effects for forming progressively distinct social engram populations.

2.3.2 Discussion

2.3.2.1 Evaluation of differential functions of dCA2 and vCA1 and their interdependency for social specification memory

Literature delineating how CA2 is implicated in social memory (Wersinger SR et al., 2002; Young WS et al., 2006; Hitti F et al., 2014; Stevenson EL et al., 2014; Smith A et al., 2016) showed how a failed intact trisynaptic circuit, where CA3 could have theoretically function to match incoming information against its "dictionary" of stored representations before conveying the output to CA1 for familiarity or novelty signaling, is proof that social recognition memory is not a mere subset of object recognition memory. The ability to distinguish a novel from a familiar stimulus is more than just a simple matching task against a stored catalog of representations for an effective recall.

Leveraging on these prior literature demonstrating that hippocampal CA2 is implicated in social memory (Raam T et al., 2017; Stevenson EL et al., 2014; Hitti F et al., 2014; Smith A et al., 2016; Lin YT et al., 2018), we show that both dCA2 and vCA1 play crucial distinct roles in a novel behavioral paradigm that tests for the recall of individual conspecifics, that were previously familiarized in separate successive epochs –referred to as the "social specification behavior paradigm". We coined the term, "social specification memory", which refers to the ability to recall multiple familiar conspecifics distinctively. The two hippocampal regions, dCA2 and vCA1, which contributes to this ability differs on several levels of comparison. Here, we focus more on what is unique pertaining to CA2.

Firstly, the differential contribution of CA2 can be attributed to its intrinsic physiological properties. At the level of genes, RGS14, which is exclusively enriched in CA2, is also known as a repressor of synaptic plasticity in CA2 pyramidal neurons. Mutant mice without this gene displayed enhanced novel object recognition memory and exhibited a faster learning curve in the Morris water maze task (Shinohara Y et al., 2012). This shows that CA2 is capable of other hippocampal-dependent behavioral learning; and suggests that the deletion of this CA2-specific gene releases the hold of suppressed plasticity in CA2 so that synaptic communication in CA2 is enhanced for learning the task. At the population level, CA2 neuronal populations have been shown to change to a larger extent as compared to neighboring regions CA3 and CA1 when subtle changes were made to contexts e.g. replacing for a new object in a familiar environment (Wintzer ME et al., 2014); and that the representations of space remap when presented with local, novel non-social or social changes to the environment (Alexander GM et al., 2016). CA2 place fields are found to appear relatively greater in number and larger in size than that in CA3 or CA1 (Mankin

EA et al., 2015), with a higher mean firing rate (Alexander GM et al., 2016; Mankin EA et al., 2015); thereby suggesting that these CA2 neurons convey relatively less spatial information than those in other CA regions. It was subsequently postulated that, because of these large place fields carry low resolution spatial information, CA2 neuronal populations could additionally code more strongly for temporal information (Mankin EA et al., 2015). Extrapolating this to rationalize our experimental finding, a comparison at the neuronal population level showed a relatively lower resolution of social specification in dCA2 than in vCA1. Perhaps this could explain why the optogenetically reactivated labeled social engrams in dCA2 did not elicit the recall of the respective familiar stimulus (Figure 1, E – H), while the opposite is true for the relatively high resolution of social specification in vCA1 (Figure 1, A – D). Hence, it is possible that CA2 neurons possess certain firing patterns that is characteristic of rate coding; and that our results of a visualized population code could likely be a "snapshot" of those firing neurons frozen in time. Nonetheless, until further investigations can verify this hypothesis (possibly using *in vivo* imaging), our data suggests that CA2 neuronal activity will likely initialize and fine-tune for increasingly segregated populations. Future studies could probably investigate whether a committed ensemble of engram neurons for a particular stimulus fire with temporal signatures that are ensemble specific.

Secondly, some intra-hippocampal connections with CA2 are unique. Sensory inputs coming in from the EC can be transmitted as such through the different fields of the hippocampus: EC → DG → CA3 → CA2 → CA1; EC → DG → CA3 → CA1; EC → DG → CA2 → CA1; EC → CA2 → CA1; EC → CA1 (Kohara K et al., 2014; Cui Z et al., 2013; Chevaleyre V et al., 2010; Dudek SM et al., 2016). Specifically, information carried by MEC and LEC layer II conveys contextual and non-spatial information such as inputs from the olfactory bulb and piriform cortex respectively (Kitamura T et al., 2015; Petrulis A et al., 2005; Chevaleyre V et al., 2010; Jones MW et al., 2011; Caruana DA et al., 2012; Kohara K et al., 2014; Mankin EA et al., 2015; Dudek SM et al., 2016; Boeijinga PH & Van Groen, 1984). The functional significance of these parallel streams of information from EC to the hippocampus remains a mystery: are coinciding inputs at CA1 simply being compared and contrasted for downstream signaling? Various studies have hinted that the hippocampus has the ability to code-switch between prospective and retrospective coding (Bieri KW et al., 2014; Zheng C et al., 2016). The fact that CA2 is situated strategically between CA3 and CA1, and that its intrinsic properties have been suggested to skew towards a temporal-, social-, and novelty-oriented spatial mapping, places CA2 in a favorable position to flexibly gate the type of information to be processed. With additional evidence of reciprocal projections (Cui Z et al., 2013) and mutual inhibition (Chevaleyre V et al., 2010; Stöber T et al., 2020) between CA2 and CA3, it supports the notion that competition exists between these two regions for a memory-based representation conveyed via CA3 during prospective coding, or a sensory-based via CA2 during

retrospective coding for transmission to CA1. It may also account for the inputs coming in from EC being shown to be preferentially re-routed directly to CA2 (Kohara K et al., 2014); hence influencing differential input signals to CA1 for further processing.

Thirdly, some extrahippocampal connections with CA2 are also unique. Some of the established inputs to CA2, which contribute to its functionally distinct characteristics: PVN of the hypothalamus (Cui Z et al., 2013; Zhang L et al., 2013) which provides vasopressinergic and oxytocinergic modulation of aggression (Pagani JH et al., 2015; Leroy F et al., 2018), and implicated in the enhancement of social memory (Smith A et al., 2016; Tirko NN et al., 2018; Raam T et al., 2001; Oettl LL et al., 2016; Lin YT et al., 2018); the SUM is suggested to provide regulation of oscillatory activity, stress response, novelty detection, and various emotionally salient situations in general (Beck CH et al., 1995; Cullinan WE et al., 1996; Choi WK et al. 2012, Cui Z et al. 2013, Kohara K et al., 2014; Ito M et al. 2009, Pan WX et al., 2002, Wirtshafter D et al. 1998); and the MS and both vertical and horizontal limbs of the DBB (Cui Z et al., 2013; Meibach RC et al., 1977; Yoshida K et al., 1995) provides cholinergic innervation; and are claimed to be involved in regulating hippocampal plasticity (Drever BD et al., 2011).

When presented with a stimulus, detecting for signs of novelty comes online wherein CA2 is facilitated by inputs from the SUM, a region prominently activated with high levels of c-Fos expression in response to novel events (Ito M et al., 2009) and for the generation of synchronous hippocampal oscillations (Kocsis B et al., 1994). It can be deduced that CA2 is selectively activated via the SUM in the presence of novel stimuli, and receives memory-based representations from CA3 together with sensory information conveyed from EC. A new CA2 neuronal population is then formed to transmit a novelty detection signal to CA1.

With both nicotinic and muscarinic receptors prominently expressed in CA2, cholinergic inputs from sources like the MS nucleus and both the vertical and horizontal limbs of the DBB are thought to influence the enhancement of attention to the presented stimulus by elevating signal-to-noise ratio (Sarter M et al. 2005). The resulting increased activity of the targeted CA2 neurons was shown to increase both hippocampal inhibition and phase locking of pyramidal neurons to theta oscillations (Dannenberg H et al. 2015).

Separately, acetylcholine also plays a role in the inhibition of CA3 recurrent network via the signaling action of muscarinic receptors (Hasselmo ME, 1999; Hasselmo ME et al., 2004); thus suggesting a

common mechanism underlining circuits involving CA2 that carry sensory-rich information will be preferentially activated with stimulation.

While dCA2 output to the LS is classically known to inhibit aggression (Brady JVet al., 1953; Wong LC et al.,2016), newer studies uncover a parallel projection circuit from dCA2 to LS that differentially resulted in distinct social behaviors in mice. dCA2 projecting to the VMHvl via the ventral LS (vLS) is associated with inhibiting aggression (Anderson DJ, 2016; Hashikawa K et al., 2016; Lin D et al., 2011; Falkner AL et al., 2016; Wong LC et al.,2016), while dCA2 projecting to dorsal LS (dLS) is associated with the opposite effect of promoting aggression (Leroy F et al., 2018). The determining factor for the more dominant pathway is hypothetically suggested to require the animal's evaluation of memories from past social experiences. Typically, the ability to discriminate and identify a novel social stimulus is likely to induce aggressive behavior as compared to that of a familiar (Connor JL et al., 1977; Szenczi P et al., 2012). In face with a novel stimulus, the circuit linking dCA2 to dLS will most likely be preferentially activated, which in turn disinhibits the VMHvl for to initiate an aggressive social attack (Leroy F et al., 2018). At this point, however, it remains unclear why this typical aggressive behavior is not usually exhibit when animals face a novel female or a novel juvenile male stimulus; but it is likely attributed to another projection activated from CA2 via the dLS to the ventral tegmental area that influences reward-seeking behavior (Luo AH et al., 2011); which in turn, might preferentially activate the CA2-vLS-VMHvl pathway to suppress aggression.

Incorporating both intra- and extra-hippocampal circuitry information and our findings in Figure 1, one could potentially posit for the following activation of projection signaling between different brain regions in a social interaction context: without a social stimulus, CA2 neurons are likely controlled by a default repressed plasticity state, and function as a substrate for time coding that is sensitive only to changes in local, but not global cues. However, when presented with a social stimulus, the following series of changes are hypothesized to ensue. The clamp on synaptic communication is released as CA2 integrates the social olfactory and associated valence signals from the olfactory bulb and piriform cortex via EC layer II's direct projection, and novelty signaling from SUM. On top of cholinergic signaling from MS and the DBB, PVN's oxytocinergic signaling will come into play to further amplify signal-to-noise ratio to increase the stimulus' saliency. CA2 neuron spatial firing will then undergo global remapping, where the initial low resolution of social, and potentially temporal, information encoded by dCA2 neurons will be further fine-tuned by downstream feedforward inhibition motifs and the OXTR-signaling pathway as it traverses into iCA2, vCA2 and then to vCA1. Thereafter, it combines with the spatial and contextual information encoded by dCA1 to be stored as a complete piece of high resolution social memory in

vCA1. Should the presented social stimulus proved to be hostile, CA2, with the regulation from perhaps PVN's vasopressinergic signaling, would then likely send signals to dLS for the animal to take on an aggression stance. Otherwise, the alternative parallel pathway of CA2 signaling to VMHvl via vLS to suppress aggression, and/or a signaling pathway to VTA via dLS for reward-seeking behavior may be activated instead. Our data provides one piece to a hypothetical circuit basis by demonstrating an interdependence of dCA2 and vCA1; hence advocating this microcircuit comprising of two functionally distinct regions of the hippocampus for the effective recall of social specification memory.

Despite the long-standing debate about whether the respective intrahippocampal segments can strictly be ascribed to differential functions – dorsal versus ventral, and comparisons among the CA fields (Cave CB et al., 1991; Nadel L, 1991; Kim JJ et al., 1992; Moser EB et al., 1998) – our finding can serve as additional evidence that the differential contributions of dCA2 and vCA1 subserve a common behavioral phenomenon despite being functionally different (Fanselow MS et al., 2010; Royer S et al., 2010; Bannerman DM et al., 2014; Kjelstrup KG et al., 2002; Ciocchi S et al., 2015).

2.3.2.2 Evaluation of "relay-like" projections within CA2 and their potential functional role in social specification memory

Our findings have demonstrated that the connectivity from dCA2 to vCA1 is a "relay-like" projection beginning from dCA2 to iCA2, iCA2 to vCA2, and then vCA2 to vCA1 (Figure 2, A – F). This finding is in contrast to another study's claim that a direct projection from dCA2 to vCA1 exists (Meira T et al., 2018). The discrepancy arising from our data is attributed to the differences in the amount of anterograde tracing virus used, and the number of injection sites done in each subject for visualization of the projection fibers using immunohistochemistry and electrophysiological studies with optogenetic manipulation. While the difference in the serotype of the virus used can be negligible as it might be compensated by the respective post-surgery down time for the subjects before experiments begin (Meira T et al., 2018: rAAV2/5 EF1a.DIO.hChR2(E123T/T159C)-eYFP; 3 weeks or more post-surgery down time; our experiment: AAV9.EF1a:DIO.hChR2(H134R).eYFP; 1 week post-surgery down time), their study used 200nl of virus, injected bilaterally, in dCA2 of their CA2-Cre (a.k.a Amigo2-Cre) mice for both immunohistochemistry visualizations of the projection and electrophysiological experiments. On the other hand, our study used 100nl in dCA2; 50nl in iCA2; and 50nl in vCA2, injected unilaterally in separate batches of our Map3k15-Cre mice. Using the specifications of 100nl, unilateral injection in dCA2, we did not find any labeled projection fiber terminating in vCA1 region of the horizontal brain slices obtained (Supplementary figure S4D; temporary example, not part of actual figure). Additionally, our electrophysiological recording data using the same injection specifications as was done in dCA2 lends support to our immunohistochemistry visualizations that there is no direct functional connection detected from dCA2 to vCA1 (Figure 2I). Therefore, it is very likely that the large volume of virus which the previous study injected into the relatively constricted area of dCA2 had leaked and extended its area of infection to probably the iCA2 and vCA2 sub-regions. As such, it will be unsurprising to find projection fibers terminating in vCA1 in their case; because the infected vCA2 would have projected fibers in vCA1 as did ours with the controlled amount of viral injection we administered into vCA2 exclusively.

To date, the discovery of a "relay-like" projection within the CA2 region is a novel finding. Each sub-region consists of excitatory, and GABA-A and/or GABA-B inhibitory neurons (Figure 2G), and these interneurons are very likely PV-positive (Erbs E et al., 2012; Botcher NA et al., 2014; Piskorowski RA et al., 2013). Studies show that CA2 inhibitory transmission is highly plastic; and this plasticity facilitates dis-inhibition (Nasrallah K et al., 2015; Nasrallah K et al., 2019) in an input-timing-dependent manner. As such, it increases CA3 signal transmission, which in turn recruits CA2 pyramidal neurons for

downstream signaling. Hence, the combined findings that CA2 pyramidal neurons has strong excitatory connections with deep CA1 pyramidal neurons (i.e. those closer to SO) (Kohara K et al., 2014), and that CA2 recruits feed-forward inhibition exclusively in deep CA1 area by means of controlling its EPSP shape and amplitude (Valero M et al., 2015; Nasrallah K et al., 2019), suggest that these CA2 interneurons potentially play a crucial role in: 1) influencing both the recruitment of similar/dissimilar populations of pyramidal neurons for information transfer to downstream deep CA1 areas, as well as 2) their firing profiles and magnitudes. Both of which may be able to explain the observations obtained in our social specification memory data (Figure 3).

Despite the extensive viral infection used to claim direct connectivity between dCA2 and vCA1 (500 nl, bilateral; Meira T et al., 2018), they do value-add to existing literature in showing the effect of CA2 PV-positive interneurons on the magnitude of vCA1 neuronal activity. As a result, their findings indirectly offer a putative mechanism to explain our findings: from CA2 to vCA1, anatomical connection exists for social-specific information transfer (Figure 1, O – R) except that we found it depicted in "relay-like" projections superimposed onto an inhibitory network within CA2 (Figure 2, D – F; Figure 3, S – T). The repeated feed-forward inhibition motif in each of the relay sub-regions within CA2 could likely contribute to "shape" the neuronal activity in vCA1. All in all, our presented discovery (Figure 2; Figure 3) propose that a feedforward inhibitory network projecting in a "relay-like" structural organization within CA2 suggests a functional role in the refining information transmission to facilitate signaling to downstream vCA1 for the formation of distinct memory engrams.

2.3.2.3 Evaluation of feed-forward inhibitory network in CA2 for social memory specification

Feed-forward inhibition is a key component for complex neural network operations in the sharpening of cognition and sensory perception (Hu H et al., 2014*;* Buzsaki G, 1984; Cayco-Gajic NA et al., 2019; Papadopoulou M et al., 2011; Stefanelli T et al., 2016). These processing systems generally involve the regulatory action from intrinsic GABAergic neurons beginning from the first relay site of sensory processing e.g. the olfactory bulb for the olfactory pathway, lateral geniculate nucleus for the visual pathway, and the cochlear nuclei in the brain stem for the auditory pathway (Brennan PA et al.,1997; Hirsch JA et al., 2015; Keating P et al., 2015). The common feature of GABAergic neuronal activity on mammalian sensory systems is that they facilitate pattern separation for a higher resolution of downstream signaling.

As examples, a sub-population of somatostatin (SST)-positive neurons has been shown to mediate feedforward inhibitory drive to the basolateral amygdala for a gain in sensory-to-valence association (BLA) (Guthman EM et al., 2020). Disruption of GABAergic inhibitory neurons in the tectal cells of pigeons affected the feedforward convergence of receptive fields underlying visual orientation selectivity (Li DP et al., 2007). Optogenetic activation of parvalbumin interneurons is shown to sharpen the tuning of orientation-selective cortical neurons in the visual cortex of the mouse, which improved stimulus discrimination (Lee SH et al., 2012). The firing patterns conveyed from olfactory receptor neurons are further shaped and refined by local inhibitory neurons from the antennal lobe in the locust (Raman B et al., 2010). In the primary auditory cortex of the adult cat, rat, and mouse, both the frequency tuning and intensity tuning profiles of excitation and inhibition are found to be similar in most cases; in which GABAergic inhibitory activity is shown to scale proportionately with the magnitude of tone-induced excitation to construct and refine auditory critical bandwidth properties (Dorrn AL et al., 2010; Froemke R et al., 2013; Wehr M et al., 2003; Tan AY et al., 2009).

The notion of such ubiquitous feedforward motif in the brain also applies to the hippocampus. An *in vitro* study showed that the feed-forward inhibition by PV interneurons narrows the window for temporal summation of EPSPs and action potential initiation in hippocampal CA1 pyramidal neurons (Pouille F et al., 2001). *In vivo* optogenetic manipulation of PV-positive interneuron shows that these interneurons regulate both the precise shape of place fields and the phenomenon of phase precession in CA1 pyramidal neurons (Royer S et al., 2012). Similar to spatial representation, Island cells in the EC tune temporal associations between multiple episodic memories through the feed-forward inhibition in the hippocampal

stratum lacunosum (SL) interneurons for regulating CA1 pyramidal neurons (Kitamura T et al., 2014). Hippocampal interneurons are also shown to be involved in generating various rhythms throughout the hippocampal structure (Gloveli T et al., 2005; Korotkova T et al., 2010; Stark E et al., 2014); and play a role in improving signal-to-noise ratio and fine-tuning of pyramidal neuron activity as well (Basu J et al., 2013; Owen SF et al., 2013; Piskorowski RA et al., 2013). Activation of OXTR-signaling pathways are also shown to augment GABAergic transmission throughout the different subfields of the hippocampus including the DG (Harden and Frazier, 2016), CA2 (Nasrallah K et al., 2015; Leroy F et al., 2017; Tirko NN et al., 2018; Nasrallah K et al., 2019), and CA1 (Zaninetti M et al., 2000; Owen SF et al., 2013; Maniezzi C et al., 2019) regions. OXTR expression and their resulting effects across interneuron subtypes is suggested, in the example of OXTR depolarizing fast-spiking hippocampal CA1 interneurons in the pyramidal layer and SO, but not affecting regular-spiking interneurons there, to fine-tune feedforward inhibition (Owen SF et al., 2013).

In CA2, majority of the interneurons are found to be delta opioid receptor (DOR)- expressing PV-positive in mice (Botcher NA et al., 2014; Piskorowski RA et al., 2013) and humans (Andrioli A et al., 2007; Benes FM et al., 1998). They are the main contributors to the strong default inhibitory control over CA2 pyramidal neurons, which receive a much higher density of PV-positive inhibitory synapses compared to neighboring CA3 or CA1 pyramidal neurons (Ribak CE et al., 1993); thereby resulting in an immense feedforward inhibition observed in CA2 with stimulation in the Schaffer Collaterals (Chevaleyre V et al., 2010). Using our social specification behavior paradigm, where the subject mice had successive epochs of interaction with either re-exposure of same mouse or with different mice in respective epochs, we were able to visualize neuronal populations correlating to stimuli in the re-exposure or different exposure sessions (Figure 3B). Re-exposures to the same mouse resulted in formations of neuronal populations with a high degree of overlap in reactivated neurons from the first interaction epoch; while exposures to different mice resulted in formations of distinct neuronal populations, correlating to different mice, with a relatively lower degree of overlap in reactivated neurons. Interestingly, extent of population segregation is not uniform throughout the entire CA2 region; but rather becomes progressively segregated beginning from dCA2, iCA2, to vCA2 to form increasingly distinct neuronal populations (Figure 3, K – L). As there is no prior data on the functional role of CA2 local interneurons in modulating such progressively specific neuronal populations, we chemogenetically disrupted the early relay stations of the inhibitory network (i.e. dCA2 and iCA2) in SLC32-Cre mice during one of the activity-dependent labeling epochs (Figure 3, M – N, P – R) to investigate the downstream effects on social specification memory in vCA1. Results show a failure to recall the ChR2-labeled familiar mouse despite optogenetic excitation in downstream vCA1 as opposed to WT controls that maintained social specification memory for both familiar mice

(Figure3, S – T). Based on the higher proportion of reactivated neurons visualized (Figure 3O), the impaired social specification memory observed in the SLC32-Cre group is likely attributed to the fact that the supposedly distinct neuronal populations correlating to different mice in vCA1 had become indistinguishable – as though they were the same mouse. Our experiments provide a circuit element mechanism to show that repeated CA2 local interneurons (plausibly PV-positive interneurons) are involved in modulating and shaping the population codes correlating to each social stimulus, such that they become progressively distinct from dorsal to ventral CA2 before being transmitted to vCA1 to be stored as separate neuronal populations for subsequent recall.

It has been suggested that the circuit mechanism for hippocampal information transmission lies in the plasticity of local inhibitory network of CA2. It mediates a dis-inhibitory increase in the recruitment of CA2 pyramidal neurons for information transfer within the hippocampus to facilitate social memory (Nasrallah K et al., 2015). This is supported by a subsequent study that conducts immediate *in vitro* recordings after an epoch of a subject's social interaction, with either a novel mouse or a familiar littermate, found that the interaction with a novel mouse resulted in a significantly smaller feedforward inhibition magnitude as compared to that with a familiar littermate (Leroy F et al., 2017). Hence, it is probable that a dampened feedforward inhibition is one way to recruit CA2 pyramidal neurons for downstream signaling. Although the data in the latter study did not test for long-term memory (i.e. 24-hour interval before novelty exposure), it provides an insight to the circuit mechanism of CA2 interneuron's modulatory effect on pyramidal neuron activity during the encoding phase of a stimulus, especially a novel stimulus.

Several questions regarding the suggested circuit mechanism remained unanswered: how is the described inhibitory modulation mechanism intrinsically social? Is the induced depression of feedforward inhibition a homogenous phenomenon throughout dCA2, iCA2, vCA2 during information encoding, or do the different relay stations employ dissimilar modulatory mechanisms at each stage for refinement of the transmitted signal (as evident from the varying degrees of neuronal population segregation)? While we may be able to attempt addressing the former in the following sub-section, the latter will continue to remain a mystery to be investigated in future studies.

2.3.2.4 Evaluation of OXTR signaling effects in CA2 on social memory specification

Neuromodulators act on distributed neural circuits to generate and store specific patterns of activity within neuronal ensembles important for behavioral performance. Blocking the activity of these neuromodulators systemically or specifically in their sensory cortex terminal fields can prevent associative and perceptual learning (Fletcher ML et al., 2002; Kroon JA et al., 2009; Letzkus JJ et al., 2011). For example, oxytocin release in the lateral central amygdala activates a subset of interneurons that inhibit pyramidal output neurons in the medial part of the central amygdala, which in turn facilitates the regulation of anxiolytic effects of oxytocin (Knobloch HS et al., 2012; Viviani D et al., 2011).

Studies that demonstrate the effects of OXTR signaling in social memory include techniques targeting ablation of OXTR using viral Cre recombinase injected into OXTR-expressing regions floxed OXTR mice to target DG, CA3a and CA2 (Raam T et al., 2017); or targeting ablation of OXTR specifically in CA2/CA3a neurons (Lin YT et al., 2018) showed impaired social discrimination in the former, and impaired long-term social memory in the latter. Sociability, non-social hippocampal-dependent memory (Raam T et al., 2017), anxiety-like behavior in the open field, elevated plus maze, and novelty suppressed feeding tests (Lin YT et al., 2018) were all unaffected in those experimental groups of mice. Therefore, these two keynote studies prove that the activation of OXTR-related pathways in the hippocampus are crucial for regulating a functional social recognition memory – parallel to findings that implicate hippocampal CA2 in social behavior (Wersinger SR et al., 2002; Young WS et al., 2006; Hitti F et al., 2014; Pagani JH et al., 2014; Stevenson EL et al., 2014; Smith A et al., 2016).

In addition to that, there are also studies investigating the mechanisms of OXT-OXTR signaling that could provide insights to the fluctuations in physiological activity recorded from excitatory and inhibitory neurons. OXTR inactivation studies revealed the involvement of both excitatory and inhibitory neurons in the hippocampus where they are expressed (Tirko NN et al., 2018; Owen SF et al., 2013). By uncovering a direct anatomical projection for OXT release into CA2 from the PVN, and then activating those oxytocinergic projections into CA2 for electrophysiological recordings, it was illustrated that the regulation of the resultant OXTR signaling pathway caused repetitive firing in CA2 (Tirko NN et al., 2018). Of those, the most relevant results for our study are probably the ones showing that the activation of oxytocinergic inputs coupled with OXTR signaling excites CA2 pyramidal neurons by likely modifying the rapid conductance with hyperpolarizing influence on resting membrane potential to

enhance excitability (Illustration 3, Figure 5G from Tirko NN et al., 2018). At the same time, OXTR signaling induced an enhancement of inhibition onto CA2 pyramidal cells by counteracting the OXT- or TGOT (OXTR agonist)-driven membrane depolarization duration; resulting in a change in burst firing patterns in CA2 with an elevated intra-burst frequency and shortened burst duration (Illustration 4, Figure 7 from Tirko NN et al., 2018). These effects may be evidence to demonstrate the synergistic effects of OXTR signaling and GABAergic inhibition to promote rapid repetitive firing in pyramidal cells (Cardin J et al., 2009). In a different hippocampal region, it was found that both CA1 pyramidal neurons and parvalbumin (PV) -positive interneurons were directly and independently responsive to the OXTR-selective agonist, TGOT, which was applied as well (Owen SF et al., 2013); suggesting that it may also share similar modulatory mechanisms as that for interneurons and OXTR signaling in CA2. Together, the combination from the two studies show that the overarching function of OXT-driven OXTR signaling culminates to a net enhancement in excitability of CA2 pyramidal cells, which then drives a bursting pattern output projected at CA1 neurons to favor excitation over inhibition; while the input from GABAergic inhibitory neurons complements the effect by increasing the intensity of the OXT/TGOT-induced bursts.

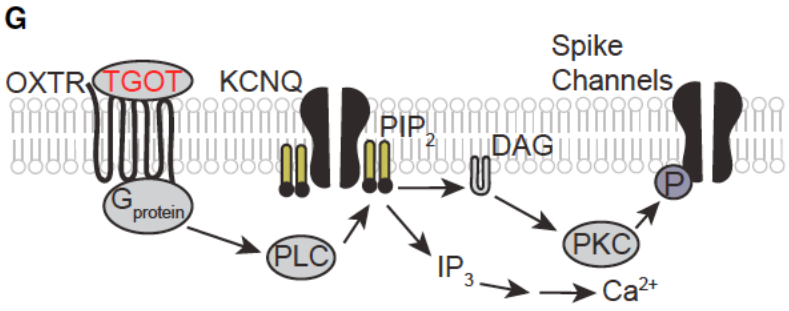

Illustration of Figure 5G, showing a proposed mechanism underlying TGOT (OXT analog) action on CA2 pyramidal neurons.

(Adapted from Tirko NN et al., 2018)

Illustration of Figure 7, showing the effects of TGOT (OXT analog) on (D) EPSP frequency in fast-spiking CA2 interneurons, (E) IPSC frequency onto CA2 pyramidal neurons, (F) TGOT-driven bursts in pyramidal neurons during control (red) and gabazine (black) conditions.

(Adapted from Tirko NN et al. 2018)

Although these findings claimed to provide the cellular mechanism between the two types of behavioral observation: the loss of social recognition function in CA2/CA3a-specific OXTR knockout mice (Raam T et al., 2017; Lin YT et al., 2018) and the loss of social recognition in CA2-silenced mice (Hitti F et al., 2014), the actual behavioral effects posited by the proposed modulation of CA2 output to CA1 due to OXTR signaling (Tirko NNet al., 2018) remains to be demonstrated. Additionally, while the findings of an amplified information transfer from dCA2 to CA1 facilitated by OXTR signaling claimed to reconcile genetic and anatomical literature specific to social recognition memory, its results are based on recordings done in dorsal CA1 (dCA1) (Owen SF et al., 2013) not ventral CA1 (vCA1); hence unable to explain for previously shown evidence of social memory being stored in vCA1, and not dCA1 (Okuyama T et al., 2016). Therefore, it remains to be proven if the suggested mechanism of an amplified, dCA2 output to vCA1 – the storage site of social recognition memory – via the effects from OXTR-signaling in both CA2 excitatory and inhibitory neurons can be demonstrated in behavior.

Our experiments attempted such an investigation by first demonstrating with loss-of-function via the ablation of OXTR to reveal that the effects of OXTR-signaling in both dCA1 pyramidal and GABAergic neurons are negligible in social recognition memory in contrast to that in CA2 and vCA1 pyramidal neurons (Figure 4, F – K). This, however, does not preclude the possibility that GABAergic interneuron activity *per se* is necessary in modulating social information processing for recall, since a reduced feedforward inhibition magnitude recorded for a novel mouse compared to that for a familiar littermate is suggestive of the important regulatory contributions from those interneurons. (Leroy F et al., 2017). With OXTR signaling shown to induce heightened intra-burst frequency of shortened burst duration in CA2

pyramidal neurons (Tirko NN et al., 2018), we hypothesized that OXTR-signaling in CA2 relay stations is necessary for forming distinct neuronal ensemble representations of multiple distinct conspecifics. Using conditional ablation of OXTR only in the early relay stations of CA2 in $OXTR^{flox/flox}$ mice, we put them through our social specification behavior paradigm for both visualization of neuronal populations, and optogenetic reactivation to test for recall of one of the familiar conspecifics in separate experiments. Despite sparing vCA2 – the sub-region that could form the highest degree of neuronal ensemble segregation in CA2 – results showed that ablation of OXTR, and hence the disruption of OXTR-signaling in CA2 excitatory neurons of early relay stations, gives rise to indistinct neuronal populations denoted by the high proportion of reactivated neurons relative to controls (Figure 4, L – N). Consequently, optogenetic reactivation of the labeled conspecific from one of the two different interaction epochs is futile since there is no committed social memory engram for that labeled conspecific to be manipulated as shown (Figure 4, O – S). Together, our results show that OXTR-signaling plays a crucial role in the circuit element mechanism of forming distinct population ensembles for the eventual recall of a particular conspecific from previously familiarized multiple ones.

2.3.2.5 Evaluation of feedforward inhibition and OXTR signaling effects (E-I contributions) for social memory specification

Thus far, we have demonstrated three main components contributing to the circuit element mechanism for social memory specification in the dCA2-vCA1 hippocampal microcircuit: 1) dCA2-vCA1 functional dependency in a "relay-like" manner from dCA2, iCA2, vCA2, to vCA1; 2) feedforward inhibition from CA2 GABAergic interneurons and 3) OXTR-signaling modulation in excitatory pyramidal neurons; where feedforward inhibition and OXTR-signaling modulation are contributive factors at every stage of the relay projection in CA2 for the formation of distinct social memory engrams for individual social stimuli. While various studies have also implicated CA2 and vCA1 respectively in social memory (Raam T et al., 2017; Lin YT et al., 2018; Hitti F et al., 2014; Stevenson EL et al., 2014; Owen SF et al., 2013; Smith A et al., 2016; Leroy F et al., 2017; Tirko NN et al., 2018), we are the first to show that the ability to recall specific individual stimuli from the social engram storage site in vCA1 is dependent on prior encoding in CA2 which fine-tunes for a segregation of respective population codes via a relay projection from dCA2, iCA2, to vCA2.

Existing literature seems to show three distinct regulatory mechanisms in CA2 in which GABAergic interneurons and OXTR signaling effects could likely shape responses of downstream pyramidal neurons: A) reduction of feedforward inhibition magnitude upon detection of social novelty (Leroy F et al., 2017; Nasrallah K et al., 2019); B) excitation of pyramidal neurons by OXTR signaling – likely via the modification of rapid conductance with hyperpolarizing influence on resting membrane potential to enhance excitability; and C) an enhancement of inhibition from interneurons onto pyramidal cells by OXTR signaling – counteracting the OXT- or TGOT-driven membrane depolarization duration to elevate intra-burst frequency and shorten burst duration (Tirko NN et al., 2018). Also, in vCA1, PV-positive neurons are shown to be implicated in the recall phase of social memory, and that they displayed higher neuronal activities when a novel mouse is approached as compared to a familiar one; thereby implying a role in social memory discrimination (Deng X et al., 2019). While it is tempting to rationalize our data by adopting the mechanisms described in those literature on the modulatory effects attributed to feedforward inhibition and OXTR signaling in inhibitory and/or excitatory neurons of CA2 (Owen SF et al., 2013; Leroy F et al., 2017; Tirko NN et al., 2018), it remains a far stretch to bridge because the experimental paradigms used in theirs and our work are different. Indeed, we are able to provide evidence that the inhibitory network is one that is feedforward in nature within area CA2; and that engram reactivation techniques provided the means to show both feedforward inhibition and OXTR-signaling induction of

enhanced excitation in pyramidal neurons are pivotal components for social memory specification in the dCA2-vCA1 hippocampal microcircuit. However, it remains a possibility that OXTR-signaling may have a modulatory effect on interneurons in CA2 to influence the disambiguation of different neuronal populations formed for social specification memory. Although not tested in our study, it is possible to probe for this by injecting a CRE virus driven by the interneuron-specific promoter, mDlx, into the early relay segments of CA2 (dCA2 and iCA2) of $OXTR^{flox/flox}$ mice; and subject them to the social specification behavior paradigm. The hypothesis here is that, when presented with the need to encode multiple conspecifics in different epochs of interaction, OXTR signaling could be engaged to modulate both the excitability of certain populations of pyramidal neurons, elevate their intra-burst frequency and shorten their burst durations via the enhancement of interneurons so that CA2 can be initialized to separately encode and hold the respective social information for transmission to downstream vCA1.

These studies also strongly suggest the notion of maintaining excitation-inhibition (E-I) balance within the microcircuit for an effective behavior readout (i.e. ability to recall a specific previously familiarized individual among other interacted, familiar individuals). The concept of a maintained E-I balance is not new. It is imperative for brains to equilibrate between excitatory and inhibitory synaptic inputs; and whose integration in the organization of neural circuits is the key for sustaining healthy normal functions (Atallah BV & Scanziani M, 2009; Wehr M et al., 2003; Yizhar O et al., 2011; Vogels T & Abbott L, 2009; Denève S & Machens C, 2016). As examples, E-I balance maintenance has been shown to be vital in: responses to varying tones in auditory cortex (Wehr M et al., 2003; Zhang LI et al., 2003), refinement of visual spatial receptive fields (Tschetter WW et al., 2018), whisker stimulation in somatosensory cortex (Wilent WB et al., 2005), during cortical up states *in vitro* and *in vivo* (Shu Y et al., 2003; Haider B et al., 2006), sensorimotor gating deficit seen in Npas4-knockout mice (Spiegel I et al., 2014; Coutellier L et al., 2012); and in various disease models such as autistic-like social behaviors, tuberous sclerosis complex, and despair-like behaviors (Gkogkas CG et al., 2013; Peca J et al., 2011; Kouser M et al., 2013; Olmos-Serrano JL et al., 2010; Gogolla N et al., 2014).

However, one common limitation to the strategy for understanding and tackling problems pertaining to E-I balance is that researchers often are manipulating either the excitatory or inhibitory component of the equilibrium state at any given point of investigation; and rarely perturbing both at the same time to mimic naturalistic simultaneous inputs coming from both excitatory and inhibitory sources. For our manipulation using the social specification behavior paradigm to probe for E-I components, additional technical difficulties that occlude a more in-depth investigation includes: disambiguating the relative contributions

of engram-specific and non-engram specific GABAergic inhibition in CA2; which of those GABAergic interneurons are co-regulated by OXT and OXTR-signaling for downstream signaling onto engram-specific pyramidal neurons; titrating for the temporal window when OXTR-signaling enhancement of excitatory activity for forming engram-specific neuronal populations occur; and recording or visualizing the combined effects of the respectively modulated excitatory and inhibitory inputs onto engram populations in each relay segment in CA2 for the progressive segregation of different social engrams. The fact that even using a double transgenic mouse expressing Cre for both CA2-specific genes and interneurons will not be ideal enough to serve our purpose; and that majority of the interneurons in CA2 are PV-positive makes it difficult to simply eyeball for patch recording, confound the problem further. Consequently, those knowledge gaps may go unsolved until the next technology advancement.

Nonetheless, we are able to show, for the first time, the pivotal roles of OXTR-signaling modulations on excitatory inputs and GABAergic inhibitory inputs in social memory and its specification – both of which regulate the appropriate balance of excitation and inhibition (E/I) in the dCA2-vCA1 hippocampal microcircuits. Both E/I imbalance and social familiarization impairments are hallmark symptoms of autism spectrum disorder (ASD) (Lee E et al., 2017), where extensive human ASD meta-analyses studies had identify OXTR as a gene heavily implicated in ASD (LoParo D et al., 2015). Our findings could potentially push frontiers to inspire the advancement of ASD treatment.

2.3.2.6 CA2 – a region for disease pathology and etiology

Given the documentation of CA2's intrinsic properties as well as its intra- and extra-hippocampal connections, it allows the speculation that CA2 is likely involved in modulating a vast variety of neuromodulators – including but may not be limited to those involving substance P, OXT, AVP, and opioids – whose alternation or disruption might attribute to disease. On top of this, the PV-positive interneurons in CA2 have been shown to undergo long-term depression with the activation of delta-opioid receptor (Piskorowski RA & Chevaleyre V, 2013); which would consequently result in an enhanced excitatory drive from CA3 to CA2 (Nasrallah K et al., 2015). Furthermore, CA2's circuitry of inhibitory neurons, which comprises of a relatively larger density of PV, somatostatin (SST), calbindin, and vasoactive intestinal polypeptide (VIP) interneurons as compared to those in CA3 and CA1 (Botcher NA et al., 2014) places CA2 in a position whereby perturbations to the delicate E-I balance of the network could potentially spin off clinical-related repercussions.

Demonstrated using mice model studies, CA2 lesions or the silencing of CA2 pyramidal neurons do result in impaired social recognition memory (Hitti F et al., 2014; Stevenson EL et al., 2014); and this gives rise to the notion that CA2 is one of the crucial regions for normal social behavior. With social behavior being one of the major symptoms seen in the spectrum of ASD patients, it then strongly supports the fact that CA2 dysfunction is possibly facilitating a fraction of its etiology. Even though further work needs to be done in order to reach a consensus on the specific brain regions or structures that underlie ASD (Bauman ML & Kemper TL, 2005), observations such as impairments especially in social memory (Boucher J et al., 2012) and a likelihood of seizures (Deykin EY et al., 1979) reported in ASD patients established a hypothesis for the involvement of the hippocampus in symptoms of ASD. Supporting that hypothesis are notable studies showing that several genes, linked to ASD in humans, are differentially expressed in lower levels CA2 compared to other hippocampal subregions in both the rodent and human brain, including *CDH10*, *CNTN3* and *SHANK3* (Shen EH et al., 2012; Hawrylycz MJ et al., 2012). However, it remains to be discovered if the expression of these genes during development stages in humans, as well as the role of CA2 in rodent social behavior, will provide a comprehensive insight to how CA2 plays a part in symptoms of ASD.

In humans, postmortem analyses have pinpointed specific alterations to CA2 and the resulting changes in its activity which were observed in patients with psychiatric disorders. Evidence include the alterations in gene expression patterns (Benes FM et al., 2007), and the specific loss of inhibitory neurons which upset the normal E-I balance equilibrium (Benes FM et al., 1998; Zhang ZJ & Reynolds GP, 2002; Narr KL et

al., 2004) – with an approximate 40% loss of those interneurons, particularly PV-positive ones, in CA2 from schizophrenic or bipolar disorder patients as compared to those in controls (Benes FM et al., 1998; Zhang ZJ & Reynolds GP, 2002; Benes FM et al., 2007, Schobel SA et al., 2013). Researchers had also successfully replicated some of the observable behavior symptoms in the spectrum of schizophrenia using animal models; and found that these symptoms are confirmed to be attributed to a reduction in CA2 PV-positive interneuron population (Berretta S et al., 2009; Piskorowski RA et al., 2016). A similar consequence of reductions in CA2 PV-positive interneurons can be seen in postmortem cases of temporal lobe epileptic patients (Andrioli A et al., 2007).

Despite the fact that disease-related symptoms and its pathology can be due to a combination of various corticolimbic structures, the unmistakable abnormalities evident in interneuron density and gene expression profiles in hippocampal CA2 seen in disorders such as ASD, schizophrenia, and epilepsy strongly suggest that CA2 is a likely pivotal locus in some of their associated symptoms.

2.3.2.7 CA2-Cortical brain regions for social memory

Apart from the anatomical connectivity between CA2 and the various subcortical brain regions that are found to be involved in a variety of social-related memory and behaviors, CA2 also receive strong inputs from cortical brain regions which in turn affects downstream transmission and processing of social information. One widely-documented cortical region with direct projections to CA2 is the MEC and LEC layers II; wherein MEC provides spatial input, while LEC provides non-spatial input which are contributed mainly from the olfactory bulb and the piriform cortex (Kitamura T et al., 2015; Petrulis A et al., 2005; Chevaleyre V et al., 2010; Jones MW et al., 2011; Caruana DA et al., 2012; Kohara K et al., 2014; Mankin EA et al., 2015; Dudek SM et al., 2016; Boeijinga PH & Van Groen, 1984). The pyramidal neurons in CA2 have also been reportedly claimed to project back to MEC layer II (Rowland DC et al., 2013); thereby possibly establishing a reciprocal projection with EC. The presence of such a direct projection route from EC to CA2, which runs in parallel alongside the neo-classical "quadsynaptic" pathway (i.e. DG→CA3→CA2→CA1) and a variant of another trisynaptic pathway bypassing CA3 (i.e. DG→CA2→CA1), is notably a projection route that provides a much stronger excitatory stimulation to CA2 pyramidal neurons as compared to that from their Schaffer Collateral inputs (Chevaleyre V et al., 2010). The presence of such a direct information transmission between CA2 and a cortical brain region could perhaps facilitate in minimizing the loss in sensory information during transmission for a more precise social recognition memory (McNaughton BL et al., 2006; Rowland DC et al., 2013) and social specification memory.

There are also other important and relevant cortical brain regions that, despite not directly connected to CA2 anatomically, are critical components of the larger "social brain network" evident from findings in both human and rodent studies.

The prefrontal cortex (PFC) has been implicated in a range of behaviors including social behavior in humans (Duncan J and Owen AM, 2000; Wood JN et al., 2003), where different regions of PFC are said to govern different categories of social cognition. For instance, the medial PFC (mPFC) region has not only been extensively to be associated to social behavior; and is also related to perceptions of others, perceptions of self and similar others (Johnson SC et al., 2002; Mitchell JP et al., 2006; Mitchell JP, 2009); the ventromedial PFC (vmPFC), which also encompasses the medial orbital cingulate cortex (mOFC) and the perigenual anterior cingulate cortex (ACC) is shown to be associated with social motivation, reward and prosocial behavior (Chevallier C et al., 2012; Meyer-Lindenberg A & Tost H, 2012; Moor BG et al., 2010; Lin A et al., 2012); mPFC and the dorsomedial PFC (dmPFC) is implicated

in acquiring, evaluating and judging mental states of self and others, moral decision making, and empathy (Amodio DM & Frith CD, 2006); and that the mPFC, vmPFC and dorsolateral PFC (dlPFC) are shown to be involved in the social cognition of perceiving, evaluating and behaving appropriately in social hierarchies where it is suggested that mPFC and vmPFC may be important for mediating appropriate behaviors given changes in hierarchies (Karafin MS et al., 2004), while dlPFC could be important for understanding the self's place in a given hierarchy (Zink CF et al., 2008). These data obtained from human studies can then be compared to that done on rodents based on the notion that the evolutionarily conserved regions of the mPFC do facilitate social behavior across species.

Regions of the human mPFC do share certain degrees of homology with regions of the rodent mPFC: the rodent prelimbic cortex (PL) is considered homologous with the human's mPFC and vmPFC regions (Riedel G et al., 2009; Wise SP, 2008); the rodent infralimbic cortex (IL) is deemed homologous with the human's vmPFC (Wise SP, 2008); the rodent mOFC is considered homologous with the human mOFC (Preuss TM, 1995); the rodent ACC is considered homologous to the human dmPFC and parts of dorsal ACC (Wise SP, 2008). Using the rodent model, earlier lesion studies done on the rodent PFC do corroborate with human data which implicates PFC in social cognition and motivation. For example, rodent OFC lesions have shown to disrupt social play behavior and increase aggression (Pellis SM et al., 2006; Rudebeck PH et al., 2007); and that ACC lesions disrupt social memory and decreases sociability (Rudebeck PH et al., 2007). To bolster the relatively crude manipulations done via lesion studies, whole brain *c-fos* activity in a social context revealed that the rodent PFC does correlatively activate given a social interaction context (Kim Y et al., 2015).

Unfortunately, there has been no compelling evidence that PFC, much less the subregions of PFC, plays a role in social recognition to date. However, given the fact that PFC is both densely interconnected amongst its subregions and connected to other cortical and sub-cortical brain regions which include nodes belonging to the "social brain network" e.g. amygdala, VTA, NAC, hypothalamus, changes to its microcircuit activity could serve to influence and/or modulate a diverse spectrum of social behaviors.

Neuromodulators such as ACh acting in the rodent PFC mediate social motivation (Avale ME et al., 2011); while OXT has been shown to mediate social affiliation via regulation of E-I balance in the auditory cortex (Marlin BJ et al., 2015) and modulation of experience-based plasticity across sensory cortices (Zheng JJ et al., 2014). Also, OXTR expressing neurons in mPFC was shown to specifically regulate for female rodent's social motivation in interacting with males (Nakajima M et al., 2014). Other factors include genetic alterations which resulted in disrupting the E-I balance, both in general and PFC-

specific, would ultimately lead to observations of ASD and schizophrenic-like symptoms. Generic gene alterations that will broadly affect social behaviors include those that manipulate changes in NMDAR expressions (Gandal MJ et al., 2012a; 2012b) and PV expression that mainly affect the interneuron population (Wöhr M et al., 2015; Lewis DA et al., 2005). On the other hand, social behaviors that have been shown to be specifically attributed to PFC dysfunction includes the demonstration of rescuing Shank3-deficient mutant mice using inhibition of cofilin specifically in the PFC, while inhibiting NMDARs in PFC is sufficient to replicate loss of social motivation (Duffney LJ et al., 2015); and that Rett syndrome mutant mice have exhibited a mPFC-specific dysfunction of excitatory transmission (Sceniak MP et al., 2015).

These findings, as a whole, demonstrate that deficiencies observed in animal models of social disorders can be attributed to perturbations to the E-I balance in the PFC which stems from neuromodulatory and/or genetic alterations. And since it has been found that several direct and indirect anatomical pathways link the hippocampus and the PFC (Barbas H & Blatt GJ, 1995; Condé F et al., 1995; Hoover WB & Vertes RP, 2007; Thierry AM et al., 2000; Rajasethupathy P et al., 2015), both PFC and the hippocampal regions (including area CA2) may then influence the neuronal population activity across both regions for a potentially amplified resultant effect to be observed on the behavioral level. Hence, it is imperative for further studies to provide more conclusive and comprehensive insights in dissociating the functional significance of PFC subregions, and their functional relationship with the various hippocampal subregions, for social memory and social behaviors in general.

2.4 Afterthoughts

By our definition, social recognition and social specification are two different processes. Social recognition is the process of simply distinguishing the novel from the familiar; while social specification can be classified as a more advanced form of recognition where the process involves distinguishing amongst multiple familiar conspecifics.

As seen in the animal world, not all of them are equally conferred the ability for processing social specification memory through evolution and/or adapting through natural selection. As examples, in the insect realm, colonial insects such as ants and bees, noncolonial insects such as the wood cricket, and others in between such as the aggregative common fruit fly learn to survive in the wild by means of social information sharing via, for example, the emissions of chemical signals (Suh GSB et al., 2004). It is an advantageous behavior in which these creatures adapt their behavior to the environment by extracting knowledge from other conspecifics (Sumpter D, 2006; Leadbeater E et al., 2007; Coolen I et al., 2005; Galef BG et al., 2005). On the other hand, for the more evolutionally-advanced insects like the queen paper wasps, they not only exhibit the ability to recognize members versus non-members of her hive; but also are able to visually discriminate amongst other familiar queens who became her "hive helpers" after having lost in battle (Tibbetts EA, 2002; Sheehan MJ et al., 2008). Other social animals, including mammals like primates, not only distinguish familiar conspecifics in their group from novel individuals from other groups, but also utilize such information to establish kinship and hierarchical relationships with the respective familiar within-group individuals. In humans, the processes of face recognition and face verification are analogous to social recognition and social specification respectively. Facial features and cues are processed via the fusiform face area for faces and the fusiform body area for body features before being transmitted to higher order processing brain regions like the hippocampus for possibly further refinement and integration into an episodic memory for retrieval when the occasion calls for it (Schwarzlose RF et al., 2005).

Our study is the first to investigate social specification memory in the hippocampus, where this process may involve different functional roles of the respective CA fields and their sub-regions, as well as different modulatory elements. By designing a novel behavior paradigm for laboratory use, we are able to provide a first glimpse in uncovering the anatomical sites for the formation and storage of social specification memory, and its underlying circuit element mechanisms underlying this neural process.

In this study, the application of the recently developed memory engram cell identification technique (Liu X et al., 2012), various cell-type specific Cre mice, and the optogenetic and chemogenetic manipulation techniques (Okuyama T et al., 2016*;* Liu X et al., 2012*;* Armbruster BN et al., 2007) enabled the demonstration that different neuronal ensembles in vCA1 store social specification memories for different familiar individuals. Although dCA2 processes social information essential for fine-tuning and transmitting relevant social specification engrams for storage in the downstream vCA1, dCA2 itself has a relatively less specific social memory at the neuronal population level. Our findings suggest that the neural code of social representations may be translated from dense population coding in dCA2, probably with rate and temporal coding, to sparse population coding in vCA1, via modulation from repeated feed-forward inhibition motif and OXTR-signaling processes.

2.5 Materials and Methods

Subjects

All experiments were conducted in accordance with U.S. National Institutes of Health (NIH) guidelines and the Massachusetts Institute of Technology (MIT) Department of Comparative Medicine and Committee of Animal Care (CAC). All animals were socially housed in a 12 h (7am - 7pm) light/dark cycle, with food and water ad libitum. BALB/cJ (Stock No: 00651), C3H/HeJ (Stock No: 00659), B6129SF2/J (Stock No: 101045), C57BL/6J, B6.FVB-Tg(Slc32a1-cre)2.1Hzo/FrkJ (Slc32a1-Cre, Stock No: 017535), B6.129(SJL)-Oxtr$^{tm1.1Wsy}$/J (Oxtr$^{flox/flox}$, Stock No: 008471), and B6J.129S6(FVB)-*Slc32a1*$^{tm2(cre)Lowl}$/MwarJ (Vgat-Cre, Stock No: 028862), were obtained from the Jackson Laboratory. Tg(Oxtr-cre)ON66Gsat, (OxtR-Cre, Stock No: 036545-UCD) was obtained from Mutant Mouse Resource and Research Centers (MMRRC). Map3k15-Cre transgenic line (Kohara K et al., 2014), Trpc4-Cre transgenic line Okuyama T et al., 2016), and Wfs1-Cre transgenic line (Kitamura T et al., 2014), were previously developed in our laboratory. All transgenic mouse lines involved in engram reactivation experiments and FT-Slow analyses were raised on a 40 mg/kg doxycycline (Dox) diet until the activated neuron labeling day. All Cre expressing mouse transgenic lines were maintained as hemizygotes. All experiments were performed using male mice.

Adeno-associated viruses (AAV)

AAV9-cfos:tTA was packaged by ViGENE Bioscience, with a titer of 1.4*10^13 genome copies/ml, which was previously described (Roy DS et al., 2016). AAV9-TRE:ChR2-EYFP (2.0*10^13 genome copies/ml) and AAV9-TRE:EYFP (1.5*10^13 genome copies/ml) were generated by and acquired from the Gene Therapy Center and Vector Core at the University of Massachusetts Medical School, which were previously described (Liu X et al., 2012). AAV9-eSARE:tTA was reproduced in the Tonegawa lab and packaged by ViGENE Bioscience, with a titer of 1.19*10^14 genome copies/ml. The pAAV-hSyn-DIO-HA-hM4D-WPRE plasmid was acquired from Bryan Roth at the University of North Carolina; and packaged as 3.3*10^12 genome copies/ml AAV-hSyn-DIO-HA-hM4D-WPRE at the University of Massachusetts Medical School Gene Therapy Center and Vector Core. AAV9-EF1a:DIO-hChR2(H134R)-EYFP (AV-9-20298P) was generated by and acquired from University of Pennsylvania Vector Core, with a titer of 1.6*10^13 genome copies/ml. pTRE:Fluorescent Timer (FT)-Slow was obtained from Addgene (Plasmid No. 31915) and TRE:FT-Slow was previously cloned in AAV vector. AAV9-TRE:FT-Slow was packaged by ViGENE Bioscience, with a titer of 1.64*10^14 genome

copies/ml, which was previously described (Okuyama T et al., 2016). The plasmid of AAV5-TRE:histoneH2B-EGFP-2A-TVA receptor-2A-rabies glycoprotein (pAAV-TRE:H2B-EGFP) was obtained from Addgene (Plasmid No. 27437) and the AAV was packaged by University of Massachusetts Medical School (UMMS), with a titer of 1.4*10^13 genome copies/ml, which was previously described (Okuyama T et al., 2016). The plasmid of pAAV-EF1a:mCherry-IRES-Cre was obtained from Addgene (Plasmid No. 55632) and packaged by ViGENE Bioscience, with a titer of 5.59*10^12 genome copies/ml. AAV5- CaMKIIα:cre-GFP was generated and packaged by University of North Carolina Vector Core, with a titer of 3.5*10^12 genome copies/ml. AAV9-hSyn:DIO-eArchT3.0-EYFP was generated by and acquired from University of Massachusetts Medical School (UMMS), with a titer of 2.5*10^13 genome copies/ml, which was previously described (Okuyama T et al., 2016). AAV9-EF1a:DIO-EYFP (AV-9-27056) was generated by and acquired from University of Pennsylvania Vector Core, with a titer of 2.93*10^13 genome copies/ml.

Stereotaxic surgery

Methods for stereotaxic surgery were described previously (Okuyama T et al., 2016). Stereotactic viral injections, microendoscope implants, and optic fiber implants were all performed in accordance with MIT's CAC guidelines. Mice were anaesthetized using 500 mg/kg avertin. Viruses were injected using a glass micropipette attached to a 10 ml Hamilton microsyringe through a microelectrode holder filled with mineral oil. A microsyringe pump and its controller were used to control the speed of the injection. The needle was slowly lowered to the target site and remained for 3 min after the injection. Three jewelry screws were placed on the skull surrounding the implant site of each hemisphere to provide additional stability. A layer of adhesive cement was applied followed by dental cement to secure the optical fiber implant. A cap made from the top part of an Eppendorf tube was used to protect the implant. The incision was closed with sutures. Mice were given 1.5 mg/kg metacam as analgesic and remained on a heating pad until fully recovered from anaesthesia. Mice were allowed to recover for 2 weeks before all subsequent experiments.

Bilateral viral delivery was used for behavioral experiments and unilateral for *in vitro* patching experiments. All coordinates are relative to Bregma: vCA1 injections were targeted to (AP: - 3.40 mm, ML: ± 3.70 mm, DV: - 4.70 mm). vCA1 implants were placed at (AP: - 3.40 mm, ML: ± 3.70 mm, DV: - 4.50 mm). dCA2 injections were targeted (AP: - 1.60 mm, ML: ± 1.60 mm, DV: - 1.70 mm). iCA2 injections were targeted to (AP: - 3.10 mm, ML: ± 3.95 mm, DV: - 3.00 mm). vCA2 injections were

targeted to (AP: - 3.00 mm, ML: + 3.10 mm, DV: - 4.70 mm). Custom vCA1 and dCA2 implants containing optic fibres (200 mm core diameter; Doric Lenses) were lowered above the injection site (vCA1, AP: - 3.40 mm, ML: ± 3.70 mm, DV: - 4.50 mm; dCA2, AP: - 1.60 mm, ML: ± 1.60 mm, DV: - 1.50 mm).

For behavioral experiments, just before the AAV injection, the AAV mixtures were prepared from stock solution. AAV9-cfos:tTA and AAV9-TRE:ChR2-EYFP mixture volume (AAV9-cfos:tTA was diluted by factor of 6 with 1x PBS; and mixed with AAV9-TRE:ChR2-EYFP in 1:1 ratio) was 200nl for vCA1. AAV9-eSARE:tTA and AAV9-TRE:ChR2-EYFP mixture volume (AAV9-eSARE:tTA was diluted by factor of 100 with 1x PBS; and mixed with AAV9-TRE:ChR2-EYFP in 1:1 ratio) was 100nl for dCA2. AAV9-hSyn-DIO-HA-hM4D-WPRE volume was 50nl for dCA2 and iCA2. For SDT (Fig. S2), the injection volume AAV9-hsyn:DIO-eArchT3.0-EYFP and AAV9-hsyn:DIO-EYFP volume were 100 nl for dCA2. AAV-EF1a:Cre volume was 200nl for vCA1. AAV5- CaMKIIα:Cre-GFP volume was 200nl for vCA1.

For *in vitro* patching experiments, the injection volume of AAV9-EF1a:DIO-hChR2(H134R)-EYFP was 100nl for dCA2, 50nl (diluted 1:5) for iCA2, and 50nl for vCA2. For showing the neural connection from iCA2 to vCA2, the 1:5 diluted AAV (AAV9-EF1α:DIO-ChR2-EYFP) was used, since injection of the undiluted virus in iCA2 showed a significant drop in cell viability in vCA2, owing to the close proximity of iCA2 and vCA2.

For FT-Slow reactivation overlap experiments, bilateral viral delivery of viral mixture comprising of AAV9-eSARE:tTA, AAV5-hSyn:DIO-H2B-EGFP and AAV9-TRE:FT-Slow (AAV9-eSARE:tTA was diluted by factor of 100 with 1x PBS; AAV5-hSyn:DIO-H2B-EGFP was diluted by a factor of 3 with 1x PBS; and both are mixed with AAV9-TRE:FT-Slow in 1:1:1 ratio). The volume for the viral mixture was 100 nl for dCA2, and 150nl for iCA2, and vCA2 respectively in Map3k15-Cre transgenic mice. AAV5-CaMKIIα:Cre-GFP volume was 150nl for dCA2 and iCA2.

Histology and immunohistochemistry

Methods for immunohistochemistry were previously described (Okuyama T et al., 2016). Briefly, adult mice were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). 50 μm brain sections were prepared using a vibratome, followed by incubation in 0.3% Triton-X PBS with 5% normal goat serum for 1 hour at room temperature (RT). Primary antibodies were added to 5% Normal Goat Serum / 0.3% Triton-X in PBS solution and incubated overnight at 4°C. All samples involving anti-RGS14 were incubated over 4 nights at 4°C. Primary antibodies: chicken anti-GFP (Invitrogen, A10262, 1:1000), mouse anti-RGS14 (NeuroMab, 75-170, 1:200), and rabbit anti-c-Fos (Santa Cruz, sc-52, 1:500). After rinsing with 1x PBS 3 times for 15 minutes each, tissue sections were incubated with Alexa Fluor-488, Alexa Fluor-546 or Alexa Fluor-633 conjugated secondary antibodies (Invitrogen, 1:500; only for RGS14 staining, 1:200) for 3 hours at RT. Sections were then washed in 1x PBS 3 times for 15 minutes and mounted using VECTASHIELD medium on glass slides. Excluding FT-slow sections, all samples were stained by DAPI (1 μg/ml) for 15 min. IHC was not performed on FT-Slow sections. Fluorescent images were taken by confocal microscopy using 10X, 20X, and 40X objectives, as well as by fluorescence microscopy using 10X, 20X objectives.

Social discrimination test (SDT)

Methods for social discrimination test (SDT) were previously described (Okuyama T et al., 2016). All behavioral experiments were performed using 12 to 20 weeks old mice, during the facility light cycle. Behavioral subjects were individually habituated to the investigator by handling for several minutes on each of two separate days (Day- -2 and Day- -1; i.e. 2 days before Day-0 and 1 day before Day-0). Handling took place in the holding room where the mice were housed. Habituation to the social discrimination chamber was performed for 10 min on each of two separate days (Day- -1 and Day-0). Social discrimination chamber is a rounded rectangle composed of 30 x 30 cm square (center part) and half circle with a radius of 15 cm on both ends (30 cm height). The subject-mice were placed in a pencil holder, with dimensions of: circle with a radius of 7.5 cm and 15 cm height. On the evening of Day-1, a subject-mouse was put into the home cage of the test-mice for 2 h familiarization, and then removed for 30 min before behavioral recording (interstimulus interval, ISI = 30 min). During the last 5 min of the ISI, the test-mouse was placed into the social chamber and allowed to explore. Behavioral recording and tracking were performed for 10 min by Ethovision XT software, using an infra-red (IR) sensitive GIG-E camera with two IR illuminators under dark conditions. For half of the behavioral assays, a novel subject-mouse was placed in the left holder and familiar subject-mouse in the right, while the remaining assays were run in a counterbalanced manner. 3-5 seconds is needed for detection of the test-mouse behavior by

Ethovision software and 9 min recording data following detection was used. Total duration during which the nose-position of test-mice was in each sniffing area, a circle centered on the pencil holder with a radius of 11.25 cm (1.5 times the holders' radius), was measured. Behavior-triggered inhibition and activation experiments were performed with minor modifications of the previously described methods (Redondo RL et al., 2014).

For optogenetic inhibition, a 561 nm green laser (561-300mW) were used for eArchT-inhibition, respectively. 15 mW (green laser) of power were delivered to the ends of the optic fiber patch-cords and constant green light was used for eArchT experiments. In the 2h inhibition experiments (Fig. S2), for test-mice and control-mice in the "Encoding Inhibition" experimental group, 10 epochs of 11 min green laser-ON followed by 1 min laser-OFF was used only during the Familiarization phase. Immediately after that, the subject-mouse was removed from the home cage of test-mice for a 30-min separation before SDT was performed for 10 min (Recall phase). For test-mice and control-mice in the "Recall Inhibition" experimental group, constant green laser-ON was given only during the Recall phase.

Optogenetic reactivation of social memory engram in vCA1 and dCA2

Mouse handling and habituation procedure were identical to SDT. All WT test-mice were raised on a 40 mg/kg doxycycline (Dox) diet until the evening of Day-1 (ON-Dox). Prior to mouse-A labeling, test-mice were given food without Dox for 48 hours for vCA1 experiments and 18 hours for dCA2 experiments (OFF-Dox) before subject mouse-A (C3H/HeJ, 6-8 weeks of age) was put into the home cage of the test-mouse for ChR2 labeling during a 2 h social interaction. Immediately after social interaction and removal of the subject-mouse A from the home cage, test-mice were returned to 40 mg/kg Dox (ON-Dox). On Day-4 (24 hours after social interaction), while still ON-Dox, a different subject mouse-B (BALB/cJ, 6-8 weeks of age) was put into the home cage of the test-mouse for a second 2 h social interaction. On Day-5 (24 hours after social interaction), SDT was performed for 10 min during blue laser stimulation (12 mW, 15 ms light pulses, 20 Hz; ON-laser group) between familiar mouse-A and a novel mouse-C (B6129SF2/J, 6-8 weeks of age). On Day-7, SDT was performed for 10 min during a 473 nm blue laser (473nm-600mW) stimulation (12 mW, 15 ms light pulses, 20 Hz; ON-laser group) between familiar mouse-B and a novel mouse-D (a different B6129SF2/J, 6-8weeks of age). For mouse-B labeling, the procedure is the same except that test-mice were placed ON-Dox on Day-2 during mouse-A interaction; and were placed OFF-Dox on Day-4 during mouse-B interaction.

For chemogenetic inhibition of CA2 neurons, clozapine-*N*-oxide (CNO, 4 mg/kg) or saline was delivered intra-peritoneally 45 minutes prior to placing subject-mouse A (C3H/HeJ, 6-8 weeks of age) or mouse-B (BALB/cJ, 6-8 weeks of age) into the home cage of the test-mouse.

FT-Slow visualization and analysis of distinct neural ensembles

Test-mice were single-housed for 2 weeks post-surgery while ON-Dox. Prior to labeling, test-mice were given food without Dox for 18 hours (evening of Day-1). In the afternoon of Day-2, a subject-mouse, mouse-A (C3H/HeJ, 6-8 weeks of age), was placed into the home cage of the test-mouse for FT-Slow labeling during a 2 h social interaction. Immediately after social interaction and removal of the subject-mouse from the home cage, test-mice were returned to 40 mg/kg Dox (ON-Dox). On Day-4 (48 hours after social interaction), test-mice were given food without Dox for 18 hours. In the afternoon of Day-5, the same subject-mouse, mouse-A (C3H/HeJ, 6-8 weeks of age), or a different subject-mouse, mouse-B (BALB/cJ, 6-8 weeks of age) was placed into the home cage of the test-mouse for a second FT-Slow labeling during a 2 h social interaction. Immediately after social interaction and removal of the respective subject-mouse from the cage, test-mice were returned to 40 mg/kg Dox (ON-Dox). 12 hours later, all test-mice were perfused. Test-mice were transcardially perfused with 4% PFA; and harvested brain samples were shaken for 2 h at room temperature. Immediately following perfusion, 50 μm brain sections were collected using a vibratome and the fluorescence images were quickly acquired by confocal microscopy without immunohistological staining. Even post-perfusion, the fluorescence of FT-Slow gradually changed from blue to red.

Both FT-Slow red and blue form-expressing cells in the respective ROIs (i.e. dCA2, iCA2, vCA2) were identified using either one of the two methods as such: 1) within the boundaries of H2B-expressing cells in Map3k15-Cre transgenic line after viral injection; or 2) staining adjacent brain slices for RGS14; and superimposing the RGS14-expressing regions to do manual cell counting. FT-Slow blue form signal in FT-Slow red form positive cells were calculated and histograms were plotted. A minimum of three coronal hippocampal slices per subject mouse for dCA2, and three horizontal hippocampal slices for iCA2 and vCA2 per subject mouse respectively were used to count the number of cells with FT-Slow blue and red form signals. All manual counting was performed blind as to the group and condition. The number of cells expressing both the FT-Slow blue and red form signals in the ROI were counted and the percentages were calculated as: (number of cells with red and blue signals) *100/ (number of cells with only red signal).

***in vitro* electrophysiological recording**

Mice were anesthetized by isoflurane and their brains dissected. Using a vibratome (VT1000S, Leica), we prepared 300-µm-thick horizontal slices in oxygenated cutting solution at ~4 °C. Slices were then incubated at ~23 °C in oxygenated artificial cerebrospinal fluid (ACSF) for 45 min to a 1 hr. The cutting solution contained 3 mM KCl, 0.5 mM CaCl2, 10 mM MgCl2, 25 mM NaHCO3, 1.2 mM NaHPO4, 10 mM D-glucose, 230 mM sucrose, saturated with 95% O_2 5% CO_2 (pH 7.3, osmolarity 340 mOsm). The ACSF contained 124 mM NaCl, 3 mM KCl, 2 mM CaCl2, 1.3 mM MgSO4, 25 mM NaHCO3, 1.2 mM NaHPO4, 10 mM D-glucose, saturated with 95% O_2 5% CO_2 (pH 7.3, osmolarity 300 mOsm). Slices were transferred to a submerged experimental chamber and perfused with oxygenated 36 °C ACSF at a rate of 3 ml min^{-1}.

Whole-cell patch clamp recordings of iCA2, vCA2 and vCA1 neurons were performed in both voltage and current-clamp mode, using an infrared differential interference contrast microscope (BX51, Olympus) with a water immersion 40× objective (N.A. 0.8), four automatic micro-manipulators (Luigs & Neumann) and a CCD camera (Orca R2, Hamamatsu). Borosilicate glass pipettes were fabricated (P97, Sutter Instrument) with a resistances of 8–10 MΩ and filled with the following intracellular solution: 110 mM potassium gluconate, 10 mM KCl, 10 mM HEPES, 4 mM ATP, 0.3 mM GTP, 10 mM phosphocreatine and 0.5% biocytin (pH 7.25, osmolarity 290 mOsm) for current clamp recordings. For voltage clamp recordings the following solution was used: Cesium methanesulfonate 117mM, HEPES 20mM, EGTA 0.4mM, NaCl 2.8mM, TEA-Cl 5mM, Mg-ATP 4mM, Na-GTP 0.3mM and 0.5% biocytin (pH 7.25, osmolarity 290 mOsm). Access resistance was monitored throughout the duration of the experiment and current clamp data acquisition was suspended whenever the resting membrane potential was depolarized above −50 mV or the access resistance drifted beyond 20 MΩ. Recordings were amplified using up to two dual-channel amplifiers (Multiclamp 700B, Molecular Devices), filtered at 2 kHz, digitized (20 kHz) and acquired using custom software running on Igor Pro (Wavemetrics). Software and code are available upon request.

Optogenetic stimulation was achieved through a 460-nm LED light source (XLED1, Lumen Dynamics) driven by TTL input with a delay onset of 25 µs (subtracted offline). Light power on the sample was 33 mW mm−2, and only the maximum power was employed. Slices were stimulated by a train of 15 light pulses at 10 Hz repeated 20 times every 6 s. EPSPs, IPSPs and action potentials were measured at resting membrane potential of the patched cell. For voltage clamp recordings, a single pulse was used to elicit EPSCs and IPSCs.

Synaptic connections were verified by taking an average of 10 to 20 individual trials from each patched neuron held in current clamp at -55mV and -75mV. If a patched cell had only EPSPs at both -55 and -75mV holding potentials, the cell was classified as one that receives only a direct excitation via light stimulation of fibers. If a patched cell showed a combination of E and IPSPs at -50mV and EPSPs at -70mV, the cell was classified as receiving both excitatory and inhibitory synaptic currents from the direct glutamatergic fibers as well as the local inhibitory interneuron. And finally, if the cell showed no PSPs in response to light stimulation at both holding potentials, it was classified as not connected. To compute the probability of connection (n successes/n tests) we used only slices with reliable ChR2 expression characterized at least by one responsive postsynaptic cell (principal cell or interneuron).

In voltage clamp recordings, cells were held either at -70mV or 0mV and a single light pulse was used to elicit EPSCs and IPSCs respectively. An average of 20 traces was acquired from each resting potential.

Most CA2 neurons were filled with biocytin, slices were recovered and fixed in 4% paraformaldehyde for post experiment verification of neurons.

Latencies were calculated from the onset of laser stimulation, rise and decay times were calculated from 20 to 80% of the response amplitude.

Statistics

Data are represented as mean ± s.e.m. All histograms include individual points, which represent the values and total number of individual samples; and also depicts the corresponding new value after an experimental treatment. What the individual samples represent is indicated within the figure legends for all experiments. Student's t-test, paired or unpaired wherever appropriate, was performed on all comparisons with exceptions being the one-way ANOVA with Tukey's multiple comparisons test between all groups in dCA2, iCA2, vCA2 for A/A re-exposures and A/B distinct exposures (Figure 3K). 95% confidence interval was used to determine significance. Significance was displayed as *P< 0.05, **P<0.01, ***P<0.001, ****P>0.0001, not significant values are denoted "n.s.". Statistical tests were performed using GraphPad Prism 7.0

2.6 Figures

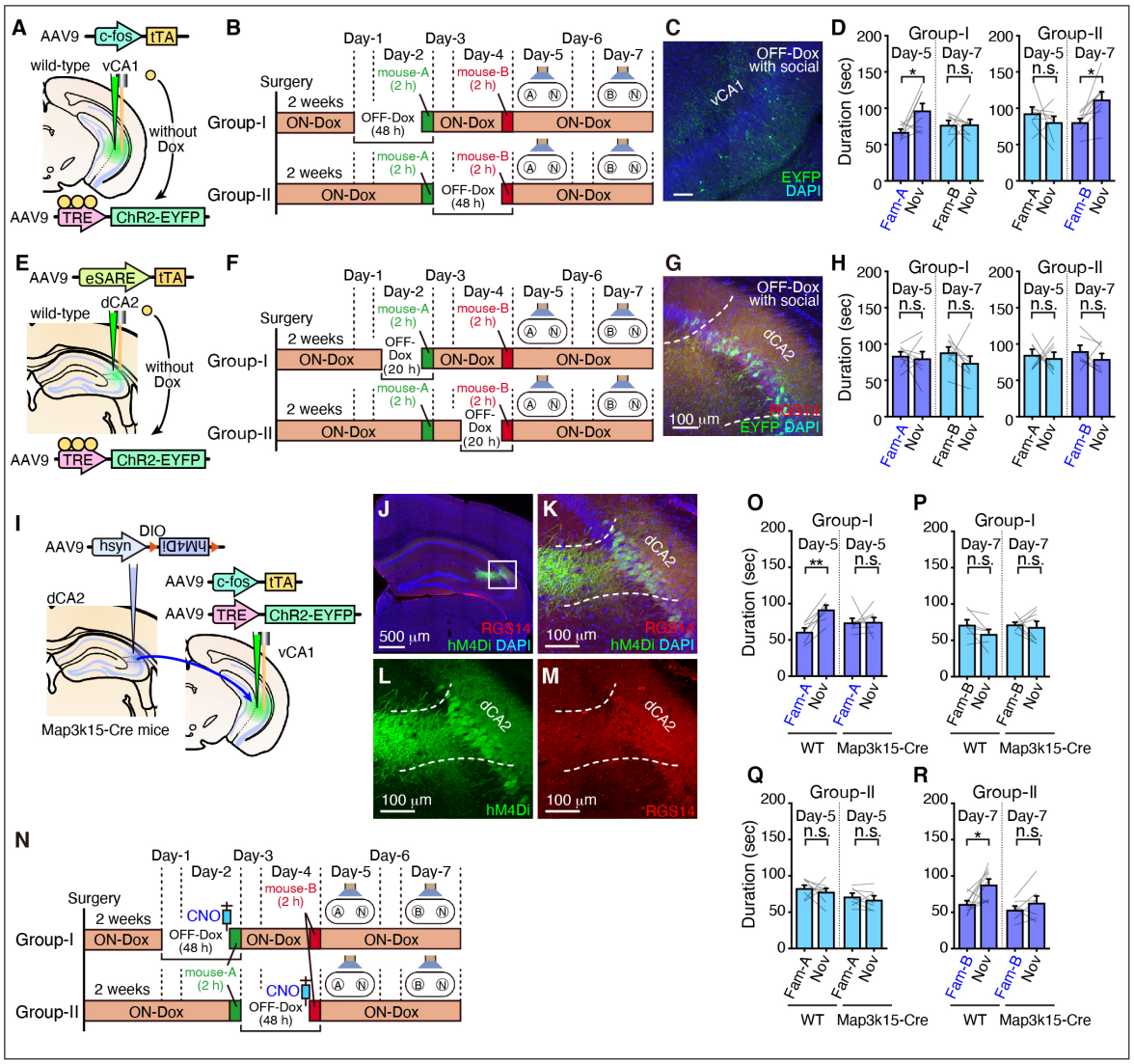

Fig. 1. vCA1 store distinct memory engrams for a pair of familiar mice.

(A – H) Opto-SDT for vCA1 and dCA2 neurons.

(A, E) Schematics of virus injection into vCA1 (A) and dCA2 (E).

(B, F) Protocols of opto-SDT for optogenetic reactivation of mouse-A engram (Group-I) on Day-5 and mouse-B engram (Group-II) on Day-7 in vCA1 (B) and dCA2 (F).

(C, G) ChR2-labeled neurons in vCA1 (C) and dCA2 (G).

(D, H) Opto-SDT of injected mice in vCA1 (D, n = 5 each) and dCA2 (H, n = 8 each). Purple, Group-I; blue, Group-II; measuring duration of social interaction on Day-5 (purple) and Day-7 (blue).

(I – R) Social information is conveyed from dCA2 to vCA1. Virus injection in Map3k15-Cre mice for dCA2 chemogenetic inhibition.

(J – M) dCA2 sections showing hM4Di-mCitrus expression, stained with anti-GFP (green), anti-RGS14 (red), and DAPI (blue).

(N) Protocol of Opto-SDT with dCA2 chemogenetic inhibition.

(O – R) Opto-SDT in Group-I (O, P; n = 7 WT; n = 8 Map3k15-Cre) and Group-II (Q, R; n = 9 WT; n = 7 Map3k15-Cre) mice on Day-5 (O, Q) and Day-7 (P, R). *$p<0.05$; ***$p<0.001$, n.s., not significant. Results show means ± S.E.M.

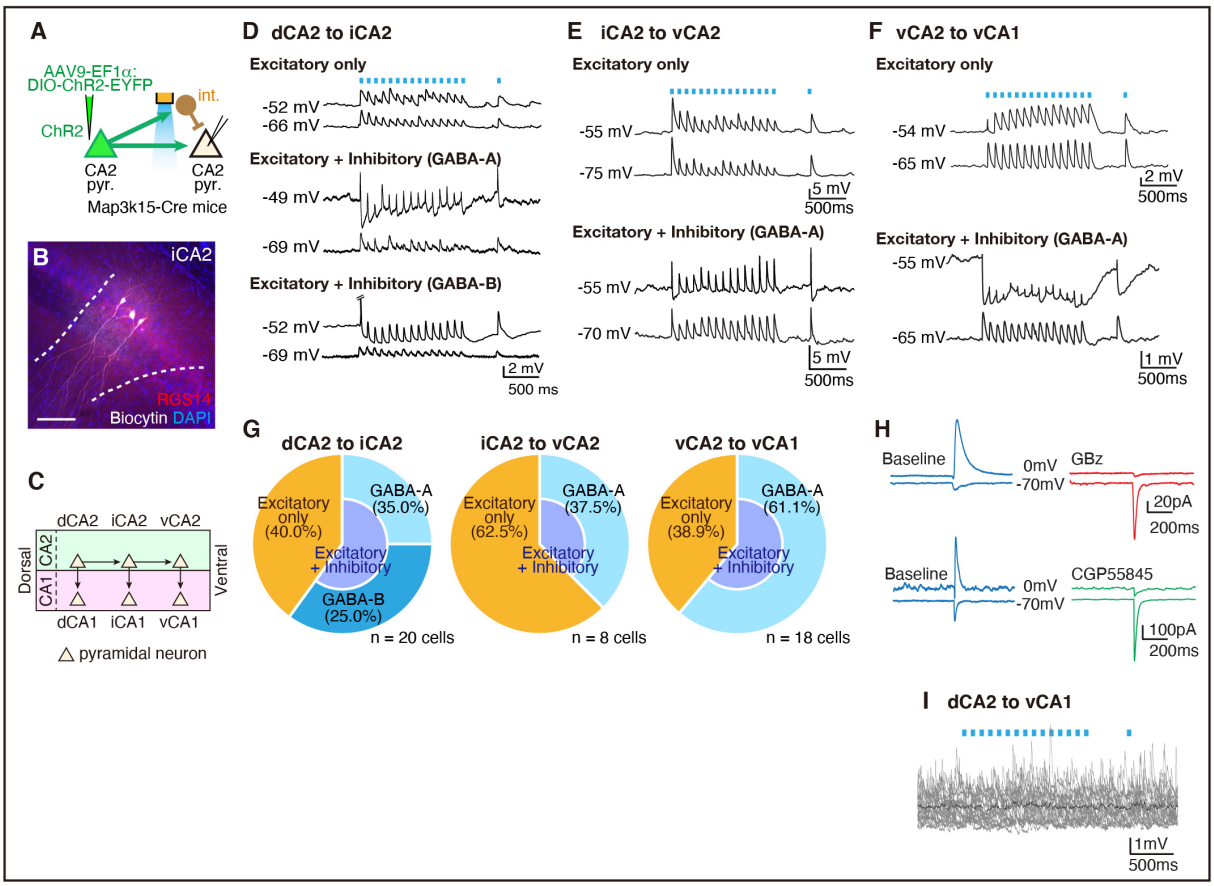

Fig. 2 Intra-CA2 connectivity and feed-forward inhibition along the dorso-ventral axis.

(A) Experimental schematic.

(B) The patched CA2 neurons filled with Biocytin (white) and stained with anti-RGS14 (red) and DAPI (blue).

(C) Schematic of circuit diagram from dCA2 to vCA1.

(D – F) Current clamp recordings of iCA2, vCA2 and vCA1 neurons with stimulation of ChR2 positive fibers of dCA2-iCA2 (D), iCA2-vCA2 (E), and vCA2-vCA1 (F).

(G) Pie charts illustrating the distribution of three types of connections.

(H) Voltage clamp traces showing currents recorded as baseline and during applications of Gabazine (GABA-A antagonist) and CGP55845 (GABA-B antagonist).

(I) Traces showing the absence of direct synaptic connections between dCA2 and vCA1.

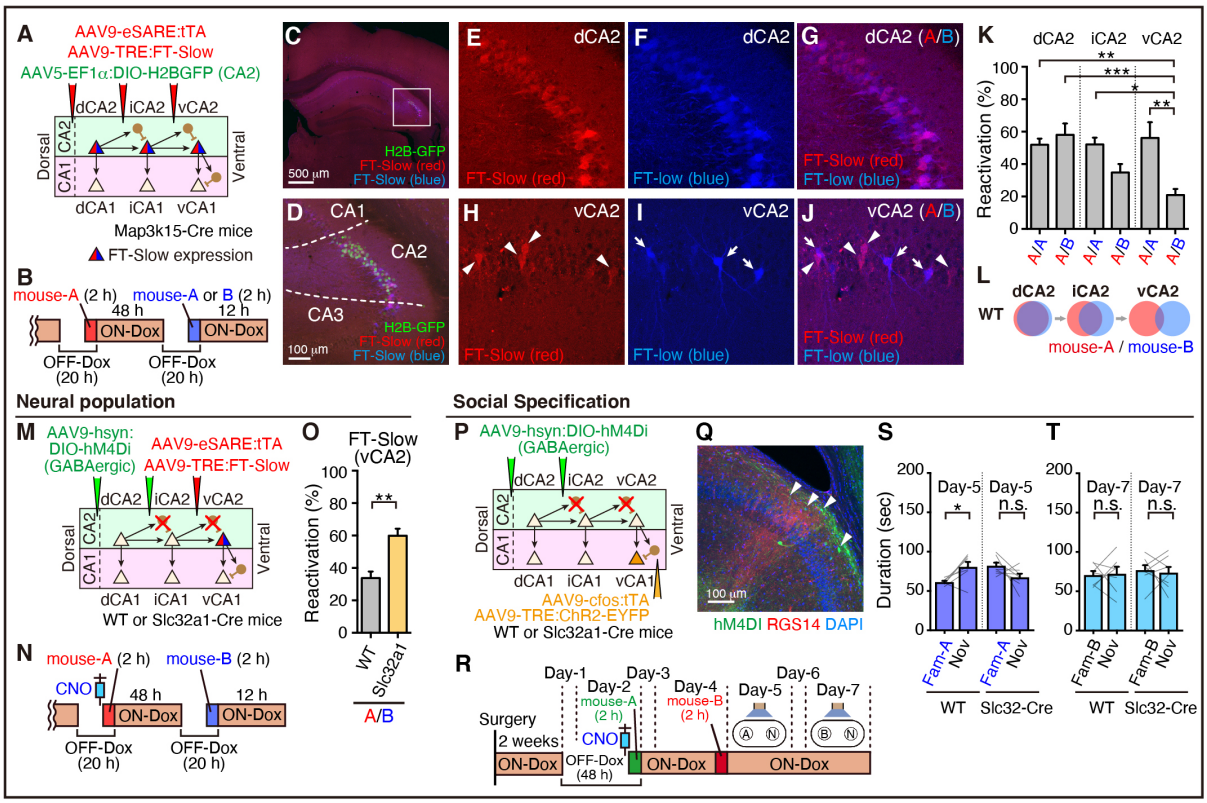

Fig. 3. Social memory specification by the repeated feed-forward inhibition.

(A) Virus injection schematic.

(B) Experimental protocol labeling mouse A- and mouse B-neurons.

(C, D) Section labeled by FT-Slow showing both red and blue forms, with H2B-EGFP expression showing CA2.

(E – J) Sections show the FT-Slow red form (E, H) representing mouse-A neurons; FT-Slow blue form (F, I) representing mouse-B neurons; and merged (G, J) in dCA2 (E to G) and vCA2 (H to J).

(K) Percentage of reactivated cells visualized in dCA2, iCA2, and vCA2 when the test mouse was exposed to the same mouse twice (A/A) or mouse A and then mouse B (A/B) (n = 6, each group).

(L) Social memory specification schematic.

(M) Schematic of virus injection.

(N) Protocol for labeling mouse A- and mouse B-neurons for chemogenetic manipulation.

(O) Percentage of reactivated cells in vCA2 with chemogenetic inhibition of GABAergic interneurons (n = 5, each group).

(P) Virus injection schematic.

(Q) iCA2 section stained with anti-GFP (green), anti-RGS14 (red), and DAPI (blue).

(R) Protocol of Opto-SDT with dCA2, iCA2 chemogenetic inhibition.

(S, T) Opto-SDT with GABAergic inhibition. (n = 7 WT; n = 8 Slc32a1-Cre). *p<0.05; ***p<0.001, n.s., not significant. Results show means ± S.E.M.

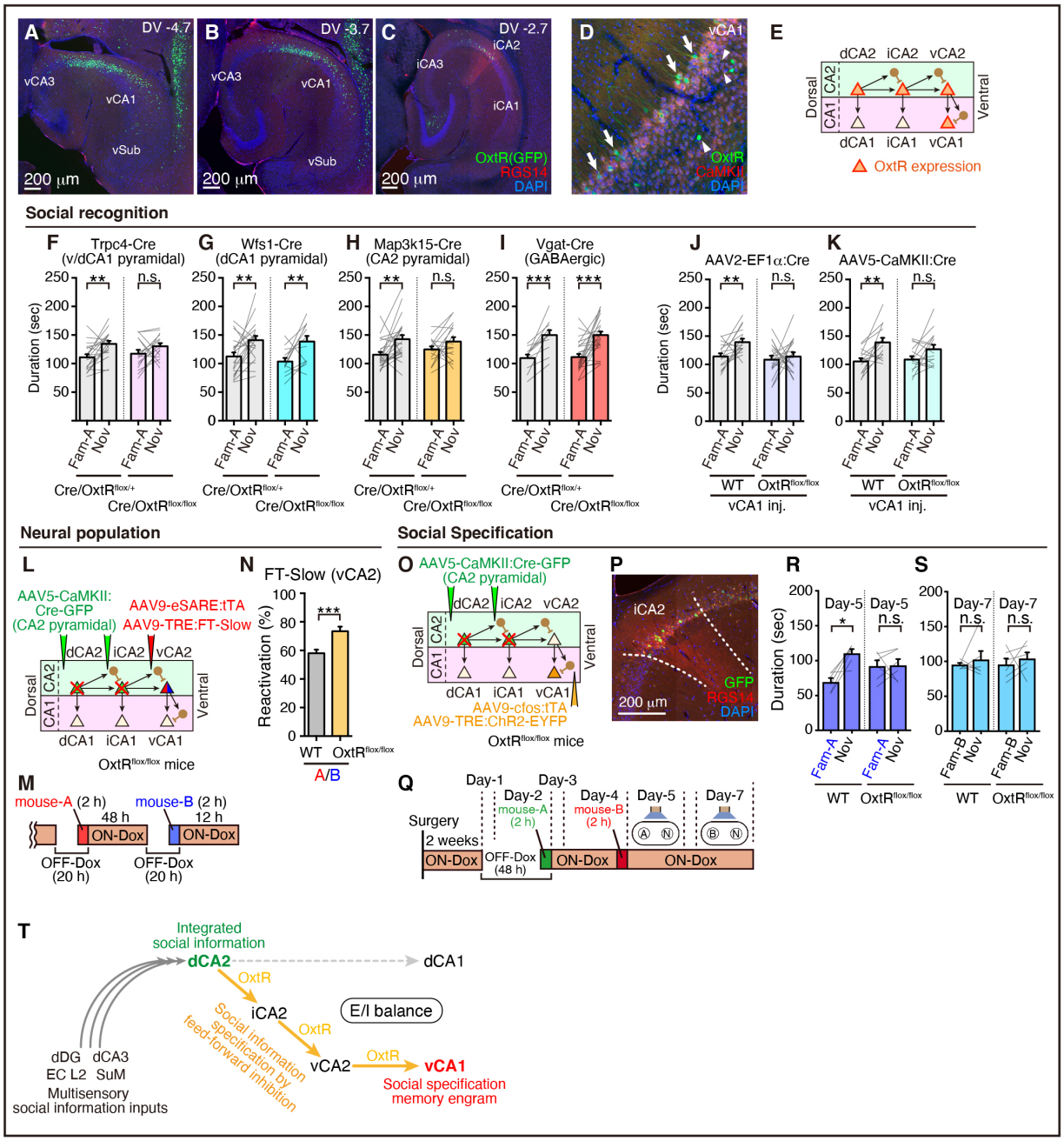

Fig. 4. Essential roles of oxytocin receptor for social recognition and specification.

(A – C) Horizontal sections of OxtR-Cre mouse injected with AAV9-hsyn:DIO-H2B-GFP, stained with anti-GFP (green), anti-RGS14 (red), and DAPI (blue). DV -4.7 mm (A), -3.7 mm (B), -2.7 mm (C).

(D) Coronal vCA1 section of (A to C), stained with anti-GFP (green), anti-CaMKII (red), and DAPI (blue) (Arrows, excitatory neurons; arrowheads, inhibitory neurons).

(E) Circuit connectivity schematic.

(F – K) SDT of OxtR $^{flox/flox}$ conditional knockout (cKO) mice and OxtR$^{flox/+}$ heterozygote mice. (F) Trpc4-Cre/OxtR$^{flox/flox}$ mice, (G) Wfs1-Cre/OxtR$^{flox/flox}$ mice, (H) Map3k15-Cre/OxtR$^{flox/flox}$ mice, and (I) vgat-Cre/,OxtR$^{flox/flox}$ mice; (J) OxtR$^{flox/flox}$ mice injected with AAV2-EF1a:Cre into vCA1, and (K) OxtR$^{flox/flox}$ mice injected with AAV5-CaMKII:Cre-GFP into vCA1.

(L, M) Schematics of virus injection and experimental protocol.

(N) Percentage of reactivated cells in vCA2 with OxtR elimination (n = 7, each group).

(O) Virus injection schematic.

(P) iCA2 section stained with anti-GFP (green), anti-RGS14 (red), and DAPI (blue)

(Q) Protocol of Opto-SDT with dCA2, iCA2 oxytocin receptor ablation.

(R – S) Opto-SDT of OxtR-cKO mice on Day-5 (R) and Day-7 (S). (n = 6 each). **p<0.01; ***p<0.001, n.s., not significant. Results show means ± S.E.M.

(T) A schematic depicting the hypothetical information flow from extra-hippocampal input sources to dCA2 for integration and fine-tuning by “relay-like” feedforward inhibitory network and OXTR-signaling modulations, and then transmitted to vCA1 to store as social specification memory engrams.

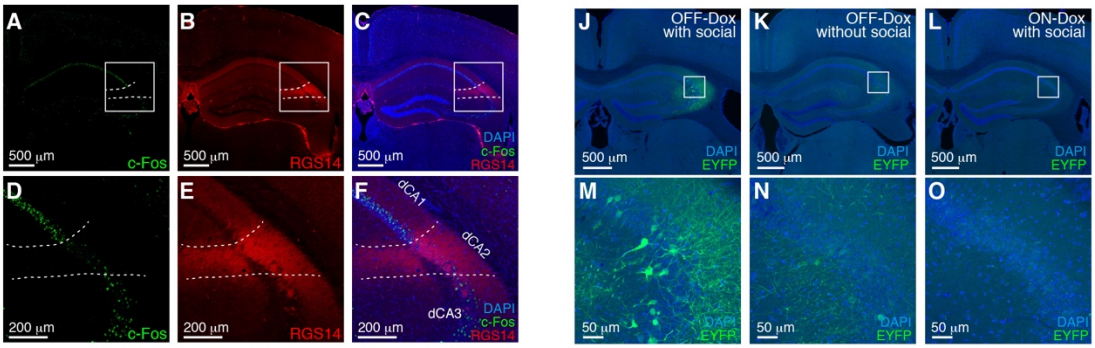

Fig. S1. Visualization of social engram neurons.

(A – F) Social interaction-induced c-Fos expression in dCA2. Coronal sections were stained with (A, D) anti-c-Fos (green), (B, E) anti-RGS14 (red), and (C, F) merged images with DAPI (blue). Little or no visible fluorescent signal from dCA2 c-Fos expression.

(J – O) Social interaction-induced levels of eSARE-driven dCA2 ChR2-EYFP expression. Coronal sections stained with anti-GFP (green) and DAPI (blue). (J, M) Social interaction during OFF-Dox. (K, N) No interaction during OFF-Dox. (L, O) Social interaction during ON-Dox.

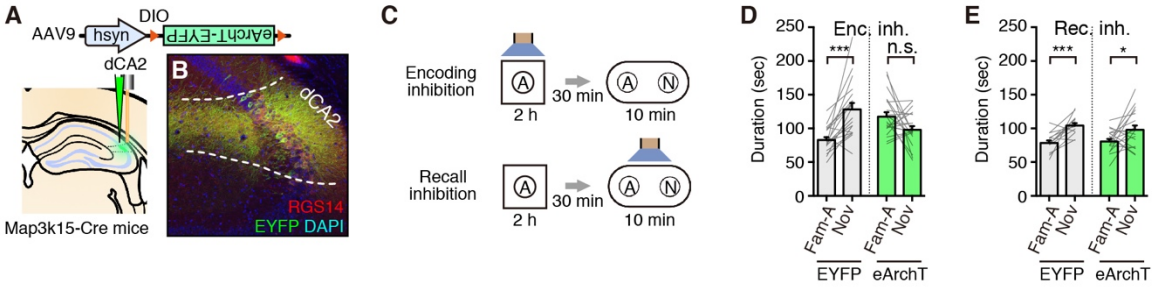

Fig. S2. Optogenetic inhibition of dCA2 neurons during the encoding and recall of social memory.

(A, B) Virus injection and optic fiber implant site in Map3k15-Cre mice for dCA2 optogenetic inhibition; and coronal section showing dCA2 eArchT expression.

(C) Protocol for optogenetic inhibition in dCA2.

(D) Encoding inhibition. SDT in control EYFP mice (grey bars, n = 15) and eArchT-EYFP mice (green bars, n = 15)

(E) Recall inhibition. SDT in control EYFP mice (grey bars, n = 9) and eArchT-EYFP mice (green bars, n = 9)

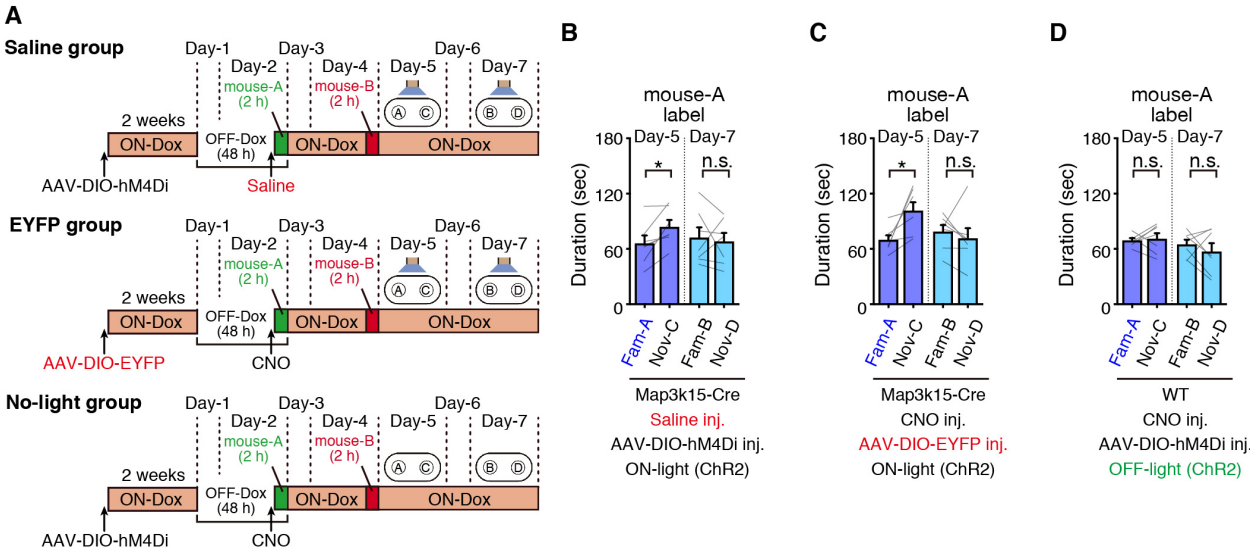

Fig. S3. Control groups of chemogenetic inhibition during the encoding phase.

(**A**) Virus injection and optic fiber implant schematic in Map3k15-Cre mice and the corresponding protocols for mouse-A specific optogenetic engram reactivation.

(**B**) SDT with engram reactivation in Map3k15-Cre mice with DREADD virus injection and Saline treatment. Purple, ChR2-labeled mouse-A engram reactivation in vCA1 shows memory recall for the familiar mice. Blue, unlabeled mouse-B cells do not result in memory recall (n = 7).

(**C**) SDT with engram reactivation in Map3k15-Cre mice with control virus EYFP injection and CNO treatment. Purple, ChR2-labeled mouse-A engram reactivation in vCA1 shows memory recall for the familiar mice. Blue, unlabeled mouse-B cells do not result in memory recall (n = 7).

(**D**) SDT without reactivation of engram cells in WT mice with DREADD virus injection and CNO treatment. Purple, memory recall fails to occur without optogenetic reactivation of ChR2-labeled mouse-A engram cells in vCA1. Blue, unlabeled mouse-B cells do not result in memory recall (n = 7).

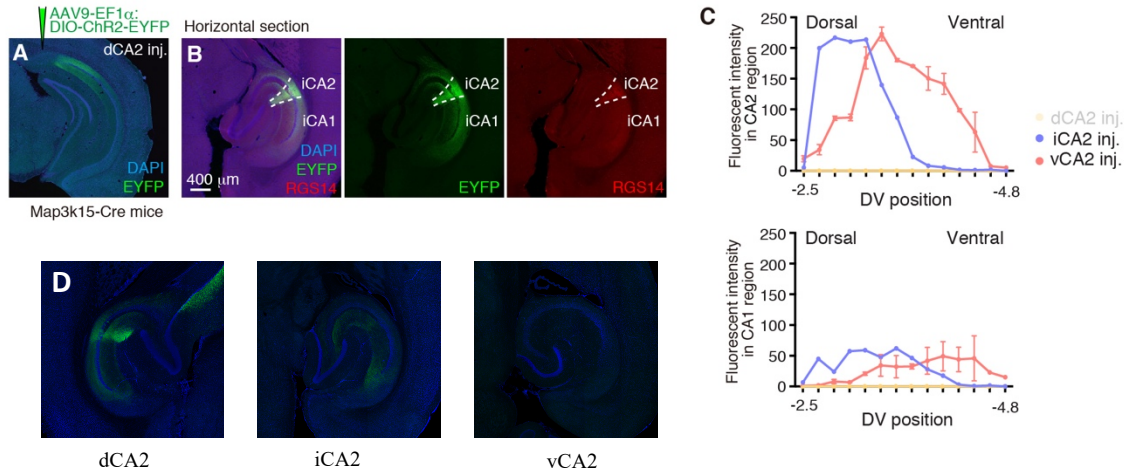

Fig. S4. Anatomical projection pattern of dCA2, iCA2, and vCA2 neurons.

(A, B) Coronal section (A) and horizontal sections (B) of Map3k15-Cre mice unilaterally injected with AAV9-EF1a:DIO-ChR2-EYFP in dCA2. Sections stained with anti-GFP (green) and anti-RGS14 (red) and merged with DAPI (blue).

(C) Quantification of immunohistological staining of ChR2-EYFP (green) expression across the DV axis (horizontal distance from DV – 2.5 mm to − 4.8 mm) of CA2 (top) and CA1 (bottom). Bars represent means; traces represent individual mice (n = 3).

(D – a temporary inclusion; not part of actual figure) Example images of anatomical projection from dCA2. Map3k15-Cre mice unilaterally injected with AAV9-EF1a:DIO-ChR2-EYFP in dCA2. Fiber projection terminates in iCA2 sections; with no visible labeled fibers detected in vCA2 sections.

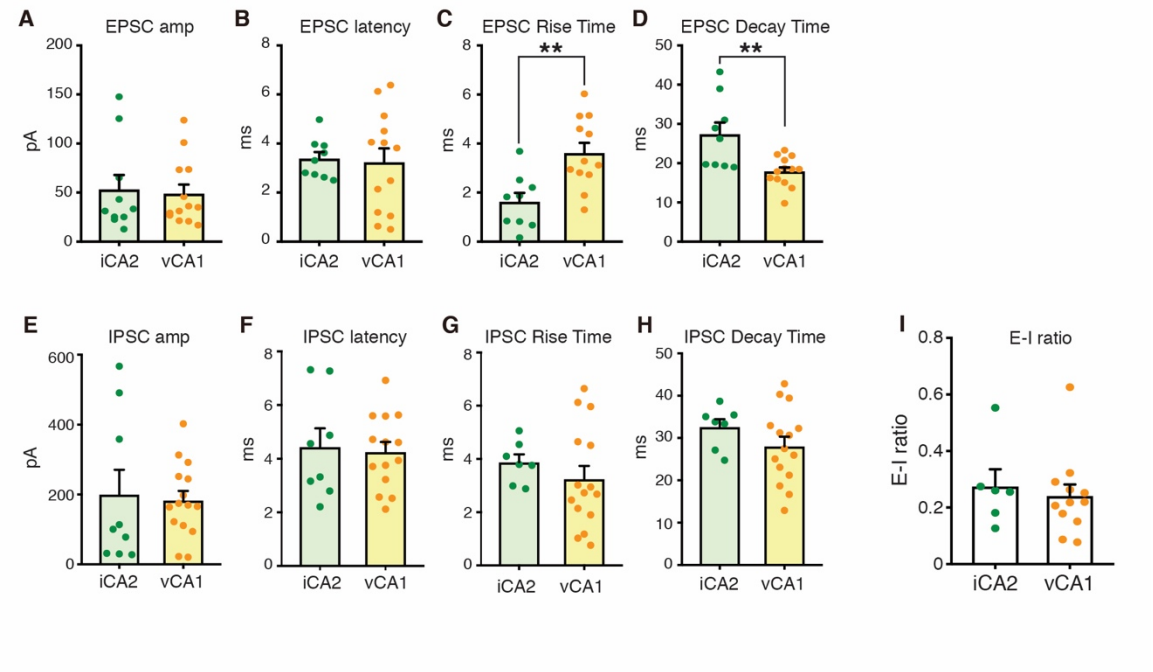

Fig. S5. Physiological characteristics of CA2 neurons.

EPSC statistics for neurons patched in iCA2 receiving projections from dCA2 and vCA1 receiving projections from vCA2.

(A) EPSC amplitudes

(B) EPSC latencies

(C) EPSC rise times

(D) EPSC decay times

(E) IPSC amplitudes

(F) latencies

(G) Rise times

(H) Decay times

(I) The calculated E-I ratio for neurons in iCA2 and vCA1.

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