Contributions of Site- and Sex-Specific LTPs to Everyday Memory

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Abstract:

Commentaries about LTP generally proceed with an implicit assumption that largely the same physiological effect is sampled across different experiments. However, this is clearly not the case. We illustrate the point by comparing LTP in the CA3 projections to CA1 with the different forms of potentiation in the dentate gyrus. These studies lead to the hypothesis that specialized properties of CA1-LTP are adaptations for encoding unsupervised learning and episodic memory, whereas the dentate gyrus variants subserve learning that requires multiple trials and separation of overlapping bodies of information. Recent work has added sex as a second and somewhat surprising dimension along which LTP is also differentiated. Triggering events for CA1-LTP differ between the sexes and the adult induction threshold is significantly higher in females; these findings help explain why males have an advantage in spatial learning. Remarkably, the converse is true before puberty: Females have the lower LTP threshold and are better at spatial memory problems. A mechanism has been identified for the loss-of-function in females but not for the gain-of-function in males. We propose that the many and disparate demands of natural environments, with different processing requirements across ages and between sexes, led to the emergence of multiple LTPs.

Keywords: Long-term potentiation, synaptic plasticity, dentate gyrus, CA1, learning, episodic memory

Introduction.

The common assertion that LTP is the substrate for memory requires qualification. The effect can be loosely defined as a sudden and lasting increase in synaptic strength induced by a brief period of afferent activity (1, 2). But a memory-related version of LTP would, in addition to the features included in the minimal definition, need to be triggered by conditions that actually occur during learning. Many of the stimulation protocols used in experimental work on activity-driven potentiation do not satisfy this requirement. The 'lasting increase' component of the definition also merits attention with regard to candidacy for LTP being a memory substrate (3, 4). Early studies of freely moving rodents showed that high frequency stimulation of the perforant path-dentate gyrus connection produces a potentiation that lasts for weeks to months (5, 6). Similarly, theta burst stimulation of the Schaffer-commissural afferents of CA1 was found to elicit stable potentiation that lasted for weeks ((7); also see (4) for review). But whether the potentiation studied in the great majority of LTP experiments is in fact long lasting remains an open question. Relatedly, efforts to arrive at general statements about the relationship between LTP and memory need to address the likelihood that there are in fact many LTPs (8-13). Plus, the possibility that different induction protocols trigger different cellular mechanisms and modifications within the same population of synapses has yet to be systematically tested (although co-existing variants have been described for the hippocampal mossy fiber synapse (14, 15)). However, as described below, studies from a number of labs have shown that there are pronounced differences in the properties of the activity-driven synaptic plasticity at the various stages of the primary hippocampal circuit. Questions about functional significance of these plasticities, thus, need to specify the locus and the particular form of LTP that is under consideration.

The LTP-memory issue is also vague with regard to the types of memory it seeks to explain. The problem is complicated by the possibility of different processes yielding seemingly similar outcomes. Operant conditioning, which has been suggested to involve LTP (16-19), has been described in a broad range of vertebrates and invertebrates (bilaterians), which suggests that some form of the effect was present in the last common ancestor of the great majority of current animals. A recent report describes operant learning by jellyfish (20), thereby implying that a version of the effect was operational even before the bilaterians. Neurons and nervous systems are radically different across the metazoan radiation and it is not likely that that the complex machinery used to produce synaptic potentiation in the mammalian hippocampus is ubiquitously distributed across this diversity. A more plausible scenario is that substrates for survival-critical operant learning evolved multiple times, somewhat in the manner proposed for eyes (21). LTP in this case would be a specialized solution – distinguished by features such as synapse specificity and rapid induction -- to a common problem. If so, then memory supported by LTP, or at least some versions of LTP, may have characteristics that distinguish it from other examples of experience-related behavioral adaptations.

This local adaptation argument raises the possibility that the learning supported by LTPs is of many types, some of which may not fit into conventional psychological categories.

Here we will evaluate evidence that a site-specific and sexually dimorphic version of potentiation plays a critical role in the encoding of information when – unlike the case for most animal studies – practice and rewards are absent. Tolman (22, 23) was among the first to argue for reinforcement-free learning as an explicit alternative to the stimulus-response, behaviorist types that dominated animal psychology for much of the 20th century. Given that the memory is formed by minimal conditions, it is reasonable to assume that its underlying mechanisms are substantially different than those used in conventional associative learning paradigms. Arguments about unsupervised learning took on greater significance when Tulving (24) advanced the persuasive argument that humans self-organize the flow of everyday experience into narrative (autobiographical) episodes. These ideas had, and continue to have, an enormous influence on research on human memory and its impairments (25). Subsequent work showed that the hippocampus plays a central role in the acquisition and retrieval of episodes (26-30). We will extend these analyses by showing that different elements of episodic memory are linked to specific sub-circuits within rodent hippocampus and that the LTP variant in one of these connections is critical to unsupervised learning and episodic memory. As will be described, this argument also relates to the profoundly important question of whether and to what degree male and females differ in how they encode the flow of episodic experience.

Site–Specific LTP in Hippocampus.

LTP was discovered in experiments using *in vivo* stimulation of the perforant path input to the dentate gyrus (DG) middle molecular layer (31), and thus likely involved the medial perforant path. Investigations into the properties and mechanisms of LTP have since focused primarily on hippocampus, including the perforant path, but with greater emphasis on Schaffer-commissural (SC) innervation of apical field CA1 stratum (str) radiatum in male rodents. LTP at this synapse, and its demonstrated reliance upon postsynaptic changes for both induction (NMDARs, calcium influx) (32, 33) and expression (F-actin remodeling, increases in synapse size and AMPAR-gated currents) (34-38), has set expectations for plasticity mechanisms at other glutamatergic synapses. Indeed, similar processes have been observed elsewhere in the cortical telencephalon (39, 40). But, as described below, recent work has shown that LTP variants quite different from that found in the CA3-CA1 connection are present at other links in the hippocampal circuit. Moreover, it now appears that the well-defined mechanisms of CA1-LTP, as elucidated in a large number of studies using male rodents, are substantially different in females. The potentiation effect is thus more differentiated, and regionally specialized, than typically thought.

Dentate Gyrus (DG): The principal afferents to the DG granule cells terminate in largely exclusive lamina within the molecular layer; these include the lateral and medial perforant paths, that respectively innervate the outer and middle molecular layers, respectively; the commissural/associational (C/A) projections generated by the hilar mossy cells that innervate the DG inner molecular layer; and a smaller input from the supramammillary hypothalamic nucleus that terminates in a thin supragranular lamina (41). Our recent studies of the lateral perforant path (LPP) demonstrated that the LPP-DG synapses express a form of LTP that is strikingly different from that in CA1. Potentiation in the LPP is triggered by NMDARs and changes in postsynaptic calcium but also requires activation of metabotropic glutamate receptor 5 (mGluR5) (12) and opioid receptor-mediated suppression of GABAergic inhibition (42, 43) (among hippocampal systems the LPP and mossy fiber systems are distinctive in containing relatively high levels of opioid peptides (44, 45)). Moreover, the same paired-pulse facilitation and AMPAR/NMDAR current ratio tests used to establish the postsynaptic localization of CA1-LTP (37) demonstrated that LPP-DG potentiation is expressed *presynaptically* by an increase in evoked transmitter release (12). The dependency of *LPP*-LTP on mGluR5 suggested a possible explanation for how potentiation could be induced postsynaptically but expressed presynaptically. Specifically, the receptor is part of a supramolecular complex ('signalosome') that includes diacylglycerol lipase α (DAGLα) and homer1. In association with calcium influx the signalosome triggers synthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG) (46) which is known to diffuse from the postsynaptic element to the cannabinoid type 1 receptor (CB_1R) on axon terminals. We confirmed that 2-AG was the retrograde signal for *LPP*-DG potentiation by showing that inhibition of DAGLα or blocking or genetically ablating the CB_1R prevented the stabilization of *LPP*-LTP (12). Moreover, treatments that elevate 2-AG levels doubled the magnitude of *LPP*-LTP whereas overexpressing the primary degradative enzyme blocked stabilization (12). Using the same techniques, we found no evidence for a critical contribution of 2-AG to LTP in CA1 or in the medial perforant path (MPP)-DG system.

Besides describing a new, site-specific form of LTP, the above results were surprising because retrograde endocannabinoid signaling is known to transiently depress transmitter release at both excitatory and inhibitory synapses (47). It follows that LPP terminals respond in a highly unusual fashion to activation of their CB_1Rs receptors. In line with this, studies using hippocampal slices showed that treatment with CB_1R agonists trigger phosphorylation of vesicular fusion protein Munc18-1 at excitatory synapses in CA1, a process that would lead to Munc18-1 breakdown and the expected reduction in evoked transmitter release (48). A similar increase in Munc18-1 phosphorylation was not evident in LPP terminals where CB_1R agonists instead increased presynaptic phosphorylation of the integrin-associated focal adhesion kinase (FAK) and RhoA kinase (12). This FAK/RhoA signaling route, which had been described for hepatocytes, provides a logical starting point for the presynaptic

cytoskeletal changes shown in parallel experiments to underlie the enhanced release that expresses *LPP*-LTP.

It is somewhat ironic to note that the substrates for LTP in the MPP, the pathway used by Bliss and Lomo to discover LTP (31), remain poorly understood. *MPP*-LTP depends on NMDARs and postsynaptic calcium, but in contrast to the LPP, it does not rely on CB_1R signaling (11). In the absence of this retrograde mechanism, a postsynaptic locus seems likely. This aligns with descriptions of other postsynaptic processes influencing LTP at the MPP-DG synapse (49, 50) including an interesting report suggesting that potentiation is associated with movement of NMDARs into the synaptic junction (51). These results provide evidence for a postsynaptic locus for *MPP*-LTP but more work is clearly needed. The same can be said for the C/A innervation of the DG inner molecular layer. These afferents from the hilar mossy cells express an NMDAR-independent, presynaptic form of LTP that persists for at least one hour (13, 52). Finally, the mixed glutamatergic/GABAergic input from the supramammillary nucleus (SuM) (53, 54) exhibits an exotic NMDAR-independent glutamatergic LTP that can be induced by simple postsynaptic depolarization without paired activation of the SuM afferents (55). This passive form of potentiation of SuM input, which is induced and expressed postsynaptically, can be triggered by theta bursts delivered to the MPP. In these instances, SuM potentiation is clearly not activated in a synapse-specific manner.

In all, tests have been made for the afferents to the four zones of the DG molecular layer with a different version of potentiation found for each; none of these correspond to the well-studied form of LTP present at CA3-CA1 synapses. What is to be made of this remarkable state of affairs? Very different types of afferents are involved, with those targeting the more proximal aspect of the dendrite being somewhat unusual for the cortical telencephalon. The DG granule cells also have many peculiar features and, from the material just discussed, this apparently extends to supporting disparate forms of synaptic potentiation. Tests for contributions of these processes to memory are lacking excepting for the perforant path. It is possible that the studies have uncovered processes that are involved in the maintenance of distinctly different types of synaptic connections but not necessarily in encoding hippocampus-dependent memories. Related to this point, and with the exception again of a small set of perforant path studies, evidence is lacking that synaptic plasticity expressed by DG afferents lasts long enough to be a substrate for anything but short-term memory.

Field CA3 and the Mossy Fibers: It is perhaps not surprising in light of the above that the peculiar terminals formed by granule cell projections into CA3 use an uncommon form of plasticity (14, 56). Early studies established that, unlike the case for SC projections, induction of mossy fiber (MF) potentiation caused a marked depression of paired-pulse facilitation (57) and therefore was presumably expressed by an increase in evoked transmitter release. Subsequent work showed that the induction of *MF*-LTP does not require NMDAR currents and relies on pre- but not post-synaptic calcium influx (14). It thus bears some resemblance to potentiation of C/A input to the DG inner

molecular layer. There is a second form of MF potentiation that involves the relatively small postsynaptic NMDAR currents at the MF-pyramidal cell synapse. This variant is induced and expressed postsynaptically by increased concentrations of membrane NMDARs triggered by mGluR5-mediated calcium store release (15). There may be points of contact between these events and mechanisms of *MPP*-LTP.

The LPP and MPP projections from entorhinal cortex continue beyond the DG to densely innervate the distal-most branches of CA3 pyramidal cell dendrites (58). Antidromic activation confirmed that the same LPP axon makes contacts on both CA3 pyramidal neurons and the outer molecular layer of the dentate gyrus. However, the endocannabinoid initiated presynaptic potentiation found in LPP>DG contacts was altogether absent in LPP>CA3 synapses. Conventional physiological tests for enhanced release in potentiated synapses proved negative and endocannabinoid receptor antagonists had little if any effect on the induction of LTP. Conversely, intracellular application of a toxin that prevents actin polymerization disrupted the stabilization of LTP in CA3 (as it does in CA1) but not in the DG (59). These results describe a rather startling instance in which two branches of the same input use very different forms of plasticity. Given the likelihood that the machinery needed to generate presynaptic LTP is transported down both branches of the LPP, we suggest that pyramidal cell spines suppress events within apposed axons terminals that are needed for enhanced release. Studies have shown that MPP-CA3 synapses express NMDAR-dependent LTP (60) but little is known about substrates. This is unfortunate because comparisons between two pathways acting at two sites could prove highly useful in extracting general rules governing the implementation of different routes to synaptic modifications.

By far the largest input to the CA3 pyramidal cells arises from within the subfield itself as a massive CA3 commissural-associational feedback system (61). This system innervates well over half of the apical dendritic field and all of the extensive basal dendrites. The apical branch of the recurrent pathway exhibits NMDAR-dependent LTP in rats (62, 63) and monkeys (64) but substrates have yet to be studied. Given that these are collaterals of the axons that form the CA3-CA1 connection, it is likely that many of the features of the well-defined CA1-LTP will be found in the CA3-CA3 synapses.

CA3-CA1: This is the site of the most complete effort to characterize LTP and define its substrates. Induction requires a significant degree of postsynaptic depolarization, NMDAR channel opening, and increases in spine calcium (1). The requirement for both afferent activity and postsynaptic depolarization ensures that potentiation only occurs at active synapses, so that other terminals on an axon or other spines on a dendrite are left unchanged. Movement of AMPARs into the synaptic zone -- a process about which much has been learned (2, 34, 65) – and a concomitant increase in EPSCs follow quickly upon the initial triggering events. Paired-pulse and AMPAR/NMDAR measurements indicate that release is unchanged (37, 66, 67). Stabilization of the potentiated state

involves multiple small GTPase-initiated signaling cascades, including activities triggered by BDNF (68, 69), resulting in reorganization of the subsynaptic actin cytoskeleton and stable expansion of the postsynaptic density (35, 36, 70, 71). The machinery involved overlaps with that used to form and modify adhesion junctions between various types of cells and it is thus not surprising to find that integrin signaling to the actin cytoskeleton plays a pivotal role (72-77). There is evidence that various other types of adhesion receptors also participate in the stabilization of CA1-LTP (78, 79). Relatedly, potentiation requires calcium-driven proteolysis (1, 80) and thus presumably replacement proteins. Results from studies using protein synthesis inhibitors have been controversial (81) but the bulk of the evidence indicates that local translation and induced gene expression are required for lasting potentiation (82-85).

The CA1 variant of LTP has proven particularly helpful in explaining the origins of various, seemingly unrelated features of memory. Examples include the following:

- Potentiation is induced with near optimal efficiency by short bursts of high frequency input spaced apart by the period of the theta rhythm (theta burst stimulation: TBS), a pattern of activity often recorded during common forms of learning (86, 87). LTP is induced by only 2 to 3 naturalistic theta bursts, which relates to the very brief periods of cue sampling needed to encode memories. CA1-LTP appears to have the lowest threshold for any lasting form of potentiation thus far tested.
- TBS induced CA1-LTP is extremely stable (4). Potentiation was shown to endure, without decrement, for weeks in chronic recording studies that used a second set of CA3-CA1 synapses to control for the stability of the stimulation-recording arrangements (7). The combination of low threshold for induction and extreme stability aligns well with requirements for a substrate for certain forms of memory.
- LTP has memory-like consolidation periods. A rapid, initial phase was discovered in experiments using cooling, anoxia, adenosine infusion or low frequency stimulation after TBS (88-90); later studies showed that rapid consolidation is dependent on actin polymerization in spines (72). Treatments that disrupt polymerization erased LTP but only when applied within 10-15 minutes of induction. A second and delayed phase of consolidation was revealed with the discovery that CA1-LTP relies on transient activation and signaling by synaptic integrins and that reactivation can only be achieved after a one hour delay. Remarkably, blocking integrins immediately prior to, but not after, their recovery eliminated previously established LTP (73, 91).
- LTP expresses a ‘spaced trials’ effect (92, 93). It has been known since the 19th century that some forms of information are more efficiently acquired when learning sessions are conducted spaced apart rather than in a single ‘massed’ trial. Numerous explanations have been offered for the effect among which is that some instances reflect the neurobiology of consolidation.

CA1-LTP exhibits a consolidation dependent, spaced trials effect. Specifically, a second TBS train doubles the magnitude of potentiation but only if it is delivered one hour after a first train (92). Two factors contribute to the effect: integrin recovery and the presence of a large population of synapses with a high plasticity threshold (91).

- The order in which afferents arrive at a CA1 dendrite determines the extent to which each will potentiate. When three small groups of fibers (A,B,C) are activated with overlapping theta bursts (B overlaps A and C overlaps B: $A_{1,2,3,4}$, $B_{\mathbf{3,4},5,6}$ $C_{\mathbf{5,6},7,8}$), then A potentiates to the greatest degree and C to the least. After LTP induction, the cue A-B-C will be more likely to drive the cell than the cue C-B-A (94). This finding could relate to the manner in which the order of the elements within a cue (e.g., phonemes within a word) is encoded by a neuron. In any event, modeling studies show that the sequence rule greatly expands the memory capacity of a CA3-CA1 type network (95).

Sex Differences in LTP.

Female but not male CA1-LTP is dependent on local estrogen: The above description of site-specific differences in LTP is based on a large collection of studies that focused almost entirely on males. There is however evidence for substantial sexual dimorphism in LTP. The rate limiting enzyme (cytochrome p450 aromatase, AROM) for synthesis of estradiol (E2), the most prevalent and potent estrogen in brain, is abundant in hippocampus and localized to axon terminals (96-99). E2 levels are several-fold higher in hippocampus than in blood in both sexes (98). Both male and female hippocampal neurons release estrogen (100). However, as first shown by Rune and colleagues (101) and corroborated by ourselves and others (98, 102), blocking local estrogen production with AROM inhibitors greatly reduces LTP in females only. Subsequent work using selective estrogen receptor (ER) antagonists, and mutants that express only the membrane or nuclear forms of ERα (103), showed that membrane ERα is critical for female LTP in CA1 (102) (**Figs. 1A,B**). In our studies of gonadally intact rats and mice, none of the ERs evaluated (ERα, ERβ, GPER1) contributed to male LTP (102, 104). Relatedly, TBS-induced activation (phosphorylation) of NMDAR-linked kinases Src and ERK1/2 and of TrkB at excitatory synapses depends on ERα in females but not in males (102) (**Figs. 1C,D**). These results suggest that in *females only* released estrogen 'boosts' kinase activation triggered by NMDAR stimulation. The ERs (α and ß) directly activate the two kinases in diverse tissues (105) and increases in synaptic phosphorylated (p) Src Y418 and pERK T202/Y204 caused by 1nM E2 infusion are dependent on ERα in females but not in males. Overall, it appears that links between ERα and LTP-critical kinases (106, 107) are better developed in females than in males, thereby enabling female use of released estrogen for synaptic modifications. Given that the NMDAR antagonist AP5 eliminates TBS-induced LTP in females as in males (34, 108, 109), we conclude that

in females both glutamate and estrogen receptors are necessary to activate kinase signaling.

The above results indicate that some aspect of LTP-related signaling is better developed in young adult males than females so that ERα-to-kinase signaling is not required. We found no sex differences in TBS-driven depolarization and NMDAR-gated synaptic currents (110). NMDARs are calcium permeant and their activation increases levels of the cation in spines, an effect that is required for LTP (34). However, recent studies suggest that NMDAR-mediated Src activation involves non-ionic coupling (111, 112); a metabotropic route has also been suggested for ERK1/2 engagement (113). Whether such effects are engaged by the minimal TBS needed to induce LTP is not known but the possibility exists that non-ionic relationships between the NMDARs and downstream kinases are better developed in males, thereby removing the need for the ERα-mediated signaling in females.

The sexually dimorphic synaptic features described above are discrete. We found no male/female differences in TBS-induced modifications of several actin management elements (e.g., β1-integrin activation, TrkB phosphorylation, cofilin phosphorylation) that stabilize LTP although, as expected, these steps were dependent upon upstream ERα in females but not males (102).

Sex differences in adult LTP thresholds: Does the addition of a local estrogen / ERα step in females have a significant effect on the characteristics of LTP? Initial tests of this possibility investigated the threshold amount of afferent stimulation needed to induce stable potentiation. Delivery of five pairs of theta bursts produced a robust potentiation of male CA3-CA1 synapses that showed no signs of decreasing in magnitude over a one-hour testing period (102). In contrast, the same stimulation applied to hippocampal slices from non-proestrus females failed to produce a measureable degree of potentiation. Moreover this paired burst stimulation increased the percentage of postsynaptic densities associated with dense concentrations of pERK1/2 in the CA1 field of activated CA3 fibers in males but not in females (102). These results suggest that the more complex machinery used by females to adjust synaptic strength is associated with an elevation in the threshold for LTP.

Sex differences in LTP reverse from before to after puberty in rodents: Sex steroid levels increase dramatically with puberty (114) raising the possibility that the estrogen-dependent CA1-LTP in females would be weaker, or exhibit a higher threshold, before vs. after puberty. We tested this using SC stimulation that was near threshold for inducing LTP in adult males: i.e., four trains of 3 theta bursts with 90 sec between trains. Contrary to our predictions, this stimulation elicited robust LTP in prepubescent (4-week old) females but not in adult females (110) (**Figs. 1E,F**). There is thus a loss of function during female puberty. Very different results were obtained in males: minimal TBS did not induce stable LTP in 4-week old males but was effective in young adults (110). Thus, the threshold for LTP changes in opposite directions from before to after puberty, in the two sexes (**Fig. 1G**). A reasonable explanation for the female effects came with the discovery that theta burst-induced depolarization of CA1 dendrites, and NMDAR-gated responses, are much greater before than after

puberty. Investigations into why the triggering events for LTP would decrease during this period uncovered a matching increase in GABAergic shunting of theta burst responses in the CA3-CA1 connection (110).

Quantitative immunofluorescence experiments did not detect puberty–related increases in the number of GABAergic synapses, as assessed by quantification of contacts immunoreactivity for the scaffolding protein gephyrin, in the CA1 dendritic lamina used for the LTP experiments (110). There was however a female-specific change in the $GABA_AR$ subunit profile over this period. Using fluorescence deconvolution tomography (FDT) to quantify numbers of gephyrin+ synapses associated with $GABA_AR$ subunits α2, α5 and ß1, we found that the number of GABAergic synapses with dense concentrations of α5 doubles from postnatal day (P) 28 to adulthood in females but not in males (110). Work from other groups showed that α5-$GABA_ARs$ potently shunt NMDAR-gated currents evoked by CA3 input (115). As predicted from these results, a negative allosteric modulator for α5 containing $GABA_ARs$ increased the size of theta burst responses and lowered the LTP threshold in adult females back to the low levels present immediately before the onset of puberty (110) (see below). In line with the observed weaker inhibition prior to puberty, the α5 modulator did not enhance the theta burst responses or LTP in slices from 4-week old females. These results indicate that age-related increases in inhibition, mediated by α5-containing $GABA_ARs$, are a contributor to the increase in LTP with late maturation.

The following section reviews evidence that rodents utilize basic elements of episodic memory and that these elements are differentially processed by various hippocampal pathways. We will then consider the argument that the distinguishing characteristics of the sexually dimorphic *CA1*-LTP are particularly appropriate for the encoding of unsupervised experience and complex episodes.

Hippocampal circuits differentially process aspects of unsupervised learning:

Perhaps the most common example of unsupervised learning (USL) by rodents involves interaction with a novel environment. The animals progressively decrease exploration over a matter of minutes and search less when returned on the following day, indicating that day one experiences had been converted into long-term memories. The widely used Object Location Memory (OLM) paradigm, which tests for recognizing the relocation of one of two identical objects, constitutes a second version of USL. Recently, there have been a number of efforts to test if rodents exhibit the much more complicated episodic learning, which is notable for having a temporal dimension (116, 117): Events can be widely spaced apart in an episode, as when walking across a campus (118), or occur in rapid succession as in a movie (119). This flexible use of time is especially notable given the prominent role played by temporal contiguity in conventional learning theories. Little is known about the factors occurring during or shortly after an episodic experience that promotes encoding but emotion may

have a positive effect (120). There is also evidence that striking and unexpected input promotes storage of the *preceding* sequence of events ('flashbulb memory') (121).

While not all aspects of episodic memory will be accessible to rodents, recent work suggests that learning paradigms including following features can be used to approximate the human phenomenon:

- Multiple commonplace cues or events;
- First time encounters with a particular collection of cues;
- Unsupervised learning – single session with no overt rewards or particularly salient cues;
- Encoding of information about the identity, location, and sequence of the cues ('what', 'where', and 'when');
- Assembling cues that are separated by either short or longer intervals into a sequence;
- Novelty and emotion to promote transfer into long-term memory;
- Association of an episode and its contents with the context in which they were experienced.

To assess this form of learning we developed episodic learning paradigms that include most of the features on the above list (121, 122). Each paradigm entails one time presentation of multiple cues (odors) followed by a retention trial that relies upon the animal's native tendency to preferentially explore a novel, or least recently experienced, cue (**Fig. 2A**). Both mice and rats acquired information about 'what', 'where', and 'when' during a first time unsupervised encounter with the cues. There was also evidence for the temporal flexibility that characterizes episodic memory. Mice recognized previously encountered odors with minutes long intervals between cue presentations or when they sampled the odors in rapid (seconds) succession during a free exploration period. They also remembered the *order* in which items were sampled whether the interval between samples was 30 seconds or 5 minutes (122). It will be noted that this last result suggests that the temporal discrimination was not mediated by a simple recency effect. Both rats and mice retain information about cue identity 24 hours after initial cue exposure in the free sampling (simultaneous presentation) version of the 'what' test but retention scores fall to chance levels by 48 hours. There was however clear evidence for retention at 48 hours when a light was flashed within five minutes of the initial cue exposure session (**Fig. 2B**) (121). In other work, we have found rodent learning in the episodic paradigms is strongly dependent on context associations (123).

The hippocampal circuitry responsible for learning the components of episodic memory has not been defined. We addressed the issue by measuring retention scores in the episodic 'what', 'where', and 'when' paradigms and using the inhibitory DREADD approach to transiently silence specific hippocampal pathways (122). Bilateral silencing of the lateral entorhinal cortex (LEC) during cue exposure thoroughly disrupted acquisition of 'what', 'where', and 'when' (**Fig. 2C**) without evident effect on performance in a simple 2-odor memory test (122). To test if episodic encoding specifically requires the LEC to DG connection (i.e., the LPP), we silenced LEC on one side and the DG on the

other, thereby sparing non-DG LEC efferents within one hemisphere. This bilateral disconnection of the LPP fully blocked encoding in episodic 'what' (52). In accord with the large body of work linking spatial information to medial entorhinal cortex (MEC), bilateral silencing of this region entirely blocked encoding of episodic 'where' but had no measurable influence on 'what' and 'when' (**Fig. 2D**) (122). These results lead to the not surprising conclusion that data about the identity of items (LEC) are critical to all aspects of an episodic memory whereas spatial information (MEC) is not required to learn cue identities or their temporal order.

Field CA3 was of interest with regard to 'when' encoding because it includes a singularly massive feedback collateral system of the type proposed by theorists to generate reverberating activity that might enable associations between items that are widely spaced in time (124). Indeed, we have shown that a 2-sec train of 5 Hz stimulation applied to the CA3 feedback collaterals produced a remarkably prolonged (minutes), self-sustained firing in ~40% of trials (122) (**Fig. 2E**). Biologically realistic simulations of CA3 suggested that such variability would occur if the pyramidal neurons underwent very large, randomly occurring depolarizations – an input arriving when a sizeable percentage of the cells happened to be partially depolarized would activate a sufficient percentage to initiate recurrent feedback within the network. Whole cell recordings confirmed that membrane potentials in CA3, but not CA1, pyramidal cells continuously undergo the dramatic (≥10mV) voltage swings predicted by the modeling (122).

Experimental work then confirmed the prediction from simulations that the CA3 network with its dense interconnectivity constitutes a complex system and as such is prone to catastrophic failure. We exploited this feature to test if depression of reverberating CA3 activity affects acquisition of cue sequences. Specifically, an AAV mediating inhibitory Gi-DREADD expression was injected into a small span of CA3 pyramidal cells in one hemisphere (**Fig. 2F**) to depress cycling activity in the bilateral network. Administration of the DREADD agonist CNO prior to initial odor sampling did not reduce retention scores on the 'what' and 'where' tests but eliminated the discrimination between cues on the basis of their temporal order in a sequence ('when') (**Fig. 2G**) (122).

These results reveal an unexpectedly selective association between the elements of episodic memory and sub-circuits in hippocampus: the MEC/MPP system is critical for 'where' encoding but not for episodic 'what' and 'when', whereas the recurrent CA3 network is needed for acquisition of episodic When but not for encoding 'what' and 'where' information.

CA1-LTP is required for encoding of unsupervised learning:

Episodic memory is encoded quickly, can persist for years (albeit in a malleable form), and often contains enormous amounts of information. As noted, LTP, as found in field CA1, expresses features that align with these points: it develops within seconds, lasts for weeks (at least), and is synapse specific. This last property, combined with empirically derived timing rules, results in

tremendous storage capacity: a capability for adding new information without disturbing synaptic changes associated with earlier material. The correspondences between biological and psychological characteristics strongly suggest that the two levels of phenomena are closely related. Largely due to the simplicity of task execution, testing the argument has often used the Object Location Memory (OLM) paradigm that has features in common with those used to assess episodic learning. Animals are given a brief, one time exposure to cues during which sampling is unsupervised (no rewards). Learning is context sensitive and dependent on hippocampal field CA1 (125). However, the episodic features of multiple, distinctly different cues and temporal ordering are not included. OLM might thus be thought of as a partial version of an episodic memory task.

It was noted earlier that the unusual behavior of synaptic integrins following their activation by TBS adds intriguing features to the stabilization of LTP that lead to non-intuitive predictions about memory consolidation. One such prediction, based on evidence for strict spacing rules for enhancement of the magnitude of LTP (92, 93) with successive rounds of stimulation (**Fig. 3A**), is that sampling sessions spaced apart by 60 minutes will produce much stronger memory than sessions separated by a shorter interval. The ubiquitous spaced training effect applies to problems involving practice sessions (126) and has an uncertain relationship to the short (5-10 min) unsupervised cue exposure experiences that characterize OLM and much of episodic learning. And there is nothing in the behavioral literature that would assign particular significance to a one-hour interval. In any event, mice given three 1-min long sampling periods separated by 1 hr had excellent OLM retention at 24 hours whereas those given a single 3-min sampling period did not (127) (**Fig. 3B**). In further accord with the LTP timing rules, sampling sessions spaced apart by 30-minute intervals did not enhance learning. Remarkably, three *20 sec* training sessions, again separated by 60 min, produced memory scores equivalent to those obtained with a single 5 minute exposure (127). Studies of integrin involvement in LTP uncovered a second phase of LTP stabilization that emerged between 45 and 60 minutes post-induction (73) (see above): Infusion of ß1 integrin neutralizing antisera, or agents that block protein insertion into membranes (brefeldin) during this interval -- but not afterwards -- caused already established LTP to decay back to baseline. Infusion of the ß1 antibodies, but not IgG control solution, into field CA1 starting at 30 minutes after the OLM sampling phase blocked formation of long-term memory for object positions. Thus timing rules for the substrates and magnitude of CA1-LTP led to accurate, non-intuitive predictions for OLM.

A very different type of prediction emerged from studies on the development of sex differences in LTP. As described, female rodents have a higher threshold for LTP induction than do males, and this was accompanied by a higher threshold for acquisition of episodic 'where' information (102, 110). However, female LTP threshold is much lower than that for males before puberty (**Fig. 1F**). To be consistent with the arguments for the association between LTP and encoding, the LTP threshold switch between prepubescent and adult animals would predict that females should outperform males

on spatial problems at the younger age. There is a large literature describing male advantages on spatial problems (128-130) but the LTP results point to a specific instance in which females should have much higher retention scores. Tests of this were positive. Four week old female mice had excellent 24 hr retention in an episodic 'where' paradigm whereas age-matched males scored at chance levels; the inverse was true for adult mice (**Fig. 3C**) (110). Thus, for both episodic acquisition and LTP, males undergo a marked improvement during puberty whereas females experience a loss of function. Results described above, indicated that the age-related adjustment to female LTP are due to an increase in α5-$GABA_AR$-mediated feedforward inhibition in CA1 (**Fig. 3D**). As anticipated from this, blockade of these receptors with a negative allosteric modulator for the α5 subunit restored spatial learning in adult females to levels seen before puberty (110) (**Fig. 3E**). In summary, LTP studies have made detailed predictions about memory that are not evident from other starting points and that have been confirmed in behavioral tests.

An alternative approach to testing for relationships between LTP and episodic-like memory is to ask if synaptic events associated with stabilization of the potentiated state are triggered by brief sessions of unsupervised learning. The development of techniques for measuring theta burst stimulation-induced actin signaling at individual synapses provided means for testing if learning produces similar effects. Initial studies showed that exploration of an open field causes a small but significant NMDAR-dependent increase in the percentage of synapses containing dense concentrations of p-cofilin in the apical dendrites of field CA1 (131). Inactivation of the constitutively active cofilin via phosphorylation is a penultimate step towards the actin polymerization that serves to anchor synapses in their potentiated state. Synapses with high levels of p-cofilin were significantly larger than their neighbors (35, 131). The size of postsynaptic densities correlates with number of AMPARs and thus presumably the size of EPSCs. Subsequent work found an increase in CA1 synapses associated with activated TrkB receptor for BDNF after a period of exploration (132); BDNF signaling is critical for the production of stable LTP by theta bursts (68, 133). It is reasonable to conclude that the signaling cascades required for LTP stabilization are set in motion by unsupervised learning and produce the same structural endpoints elicited by theta bursts.

Concluding Comments

The commonplace nature of unsupervised learning somewhat obscures the complexity and unusual properties of the synaptic events needed for encoding. As described, rodents sampling four different odors for about half a minute while reacquainting themselves with an arena will notice if one of those odors is missing (replaced) in tests conducted the next day. More surprising still, they apparently remember the locations for each of the odors. Other experiments strongly suggest that the animals acquire information about the temporal order in which the cues were sampled. There are clear correspondences between these effects and the episodic memory recorded in people (118), and

it is accordingly possible that this type of learning is a characteristic feature of the mammals. It is not unreasonable to assume that its acquisition was vital to the success of the group. We have argued here that there are several forms of activity driven synaptic potentiation (LTPs) in the hippocampus and that the particular version expressed by apical CA3-CA1 synapses, and possibly many sites in cortex and amygdala, was shaped by the stringent requirements for an USL encoding device. Whether the unexpected features of CA1-LTP such as delayed consolidation (73) and the efficacy of stimulation with one hour spacing (91, 92) are also adaptations or instead consequences ('exaptations') of the cell biological adjustments required to accommodate the essential features of USL is unknown. This also holds for the striking sex differences in the substrates and functional properties of the CA1. What are the advantages of a higher LTP threshold in females and, relatedly, of a reversal of male-female differences in the facility for LTP during puberty? Tests are lacking but we predict that there will be aspects of USL, and episodic learning in particular, for which slower acquisition (more sampling) is an advantage. This relates to the general idea that rapid encoding can be maladaptive in noisy environments. If so, and given the further assumption of cooperative activity between males and females, then sex differences in CA1-LTP could have circumstance-dependent benefits for social groups.

While CA1-LTP aligns well with USL, it may be less than optimal for encoding the action-reward linkages that are fundamental to operant learning. There is evidence that learning new cues in a well-trained simultaneous 2-cue discrimination problem activates LPP-LTP markers in the outer molecular layer of the DG (12) and that manipulating the retrograde signaling (spine to terminal) required for potentiation has the predicted consequences for operant learning (11). A possible interpretation of these results is that the type of LTP found in the LPP is specialized so as to be sensitive to reward signals from the brainstem. Tests of whether activation of the dopaminergic inputs to the DG lowers the LPP-LTP threshold would be of interest in this regard. Another more widely discussed role for the LPP-DG system involves sharpening the distinction between inputs that have extensive overlap in their constituent elements ('pattern separation') (134). The greater number of cells in the dentate gyrus than entorhinal cortex dictates that the projection from the latter to the former will be divergent, an arrangement recognized by Marr (1971) (135) as being conducive to pattern separation. Other work on sparse networks showed that LTP-based synaptic learning rules lead to categorization of cues, a process that necessarily involves the separation of inputs with shared features (136). More generally, the long history of work investigating hippocampal contributions to behavior suggests that the structure has multiple functional roles – if so, it would not be surprising that it utilizes multiple types of encoding devices. But relating particular instances of plasticity to global operations will require analyses of how synaptic adjustments affect circuit level operations in the hippocampus, a critically important topic about which almost nothing is known.

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Figure Captions

Figure 1. Sex differences in CA3-CA1 LTP switch over the ages of puberty. In hippocampal slices, TBS (at arrow) or low frequency stimulation (LFS) was applied to the CA3-CA1 projections and measures were collected from CA1 str. radiatum. A,B) Plots of CA3-CA1 fEPSP slopes show that stabilization of TBS-induced LTP is impaired (A) in female rat lacking membrane ERα (i.e., NOER mutants) and (B) in female, but not male, rat by infusion of ERα antagonist MPP (3μM). C,D) In slices from both males and females, TBS increases the number of postsynaptic densities (PSD95) densely double-labeled for (C) pSrc and (D) pTrkB (compared to labeling in slices receiving control LFS). Infusion of MPP attenuated (pSrc) or fully blocked (pTrkB) this increase in females only. Results are from dual-labeling immunofluorescence (shown in images) and automated fluorescence deconvolution tomography analysis (bar: 10 μm large image, 2 μm inset). E,F) Plots of CA3-CA1 fEPSPs show that (E) in slices from adult rats TBS triplet elicits stable LTP in males but not females, whereas (F) the inverse was true for slices from prepubescent rat (Prepub, 3-4 weeks old). G) Plots of the percent LTP (relative to baseline, at 55-60 min post TBS) elicited by near threshold triplet stimulation in prepubescent and adult rats of both sexes: This near threshold stimulation elicits LTP in females only before puberty and in males only in adulthood. Mean ± SEM values plotted; *p<0.05, **p<0.01, ***p<0.001. Modified from (102) for A-D and (110) for E-G.

Figure 2. Differential involvement of hippocampal systems in acquiring the different components of episodic memory. A) Illustration of paradigms used to test acquisition of cue identity ('what'), location ('where'), and temporal order ('when'): Each task entails presentation of multiple odor cues in cups with perforated lids (letters denote specific odors) either serially ('what' and 'when') or simultaneously ('where'). The control '2-odor' task assessed recognition of a single odor and the animals' ability to discriminate cues. B) Presentation of a mild strobe flash after initial odor exposure enhanced retention (recognition of novel cue) in a simultaneous odor episodic 'what' task. C,D,F,G) AAV constructs mediating expression of the inhibitory Gi-DREADD (and fluorescent tag) were injected into specific subfields to enable regional silencing with CNO treatment before behavioral testing ~4 weeks later. C) Image shows expression of the Gi-DREADD tag in lateral entorhinal cortex (LEC) and its projections into the DG (arrow): bilateral LEC silencing blocked acquisition in the episodic 'what', 'where', and 'when' tasks. D) Bilateral silencing of medial entorhinal cortex (MEC) blocked episodic 'where' acquisition only. E) A 10-pulse theta train applied to CA3-CA1 projections elicits a prolonged period of heightened field CA1 cell firing that lasts over 5 min. F) Expression of the Gi-DREADD tag in the CA3 injection site (asterisk) and projections into DG and throughout str radiatum (sr). G) Unilateral silencing of a span of CA3 blocked episodic 'when' encoding without dampening acquisition of episodic 'what' or 'where' information. Bar in panels C, F= 400μm. Data presented are mean ± SEM. *p<0.05, ***p<0.001. Modified from (122) and (121).

Figure 3. The status of CA1 and LPP-LTP predict performance in object location memory (OLM) and episodic memory tasks. A) When spaced by 60 mins, two rounds of TBS (TBS1,TBS2) each elicit comparable enhancement of the CA1 fEPSP; if spaced by 10-40 mins TBS2 has no effect (91). B) In the OLM task mice given a single 3-min object ('massed') exposure fail to learn object location, whereas mice given three 1-min trials ('spaced'), spaced by 60 min, learn in this field CA1-dependent task (127). C) In line with age effects of CA3-CA1 LTP (in Fig. 1G) prepubescent females outperform adult females (and males) in an episodic 'where' task; in contrast males fail to learn in this task prior to puberty but exhibit robust learning in adulthood. D,E) In line with evidence that increased inhibition dampens but CA1-LTP and learning in females, treatment with a negative allosteric modulator for the α5 $GABA_AR$ subunit (L655,708, 'L655') lowers the threshold for female CA1-LTP (D) and learning (E) in the OLM task. Mean ± SEM values shown; *p<0.05, **p<0.01, ***p<0.001. From (110).

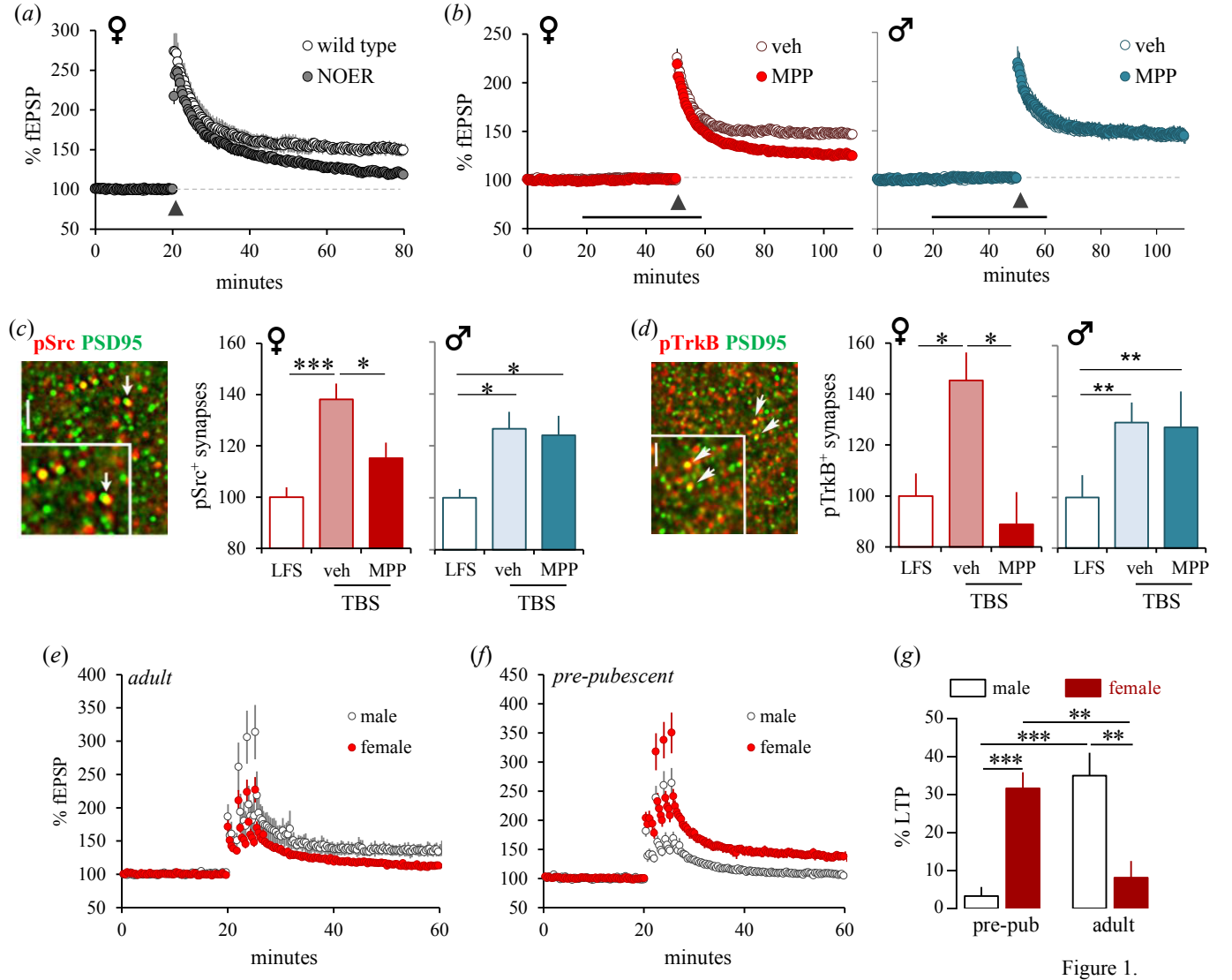
(a)
% fEPSP
minutes
wild type
NOER
(b)
veh
MPP
(c)
pSrc PSD95
pSrc+ synapses
LFS
veh
MPP
TBS
(d)
pTrkB PSD95
pTrkB+ synapses
(e)
adult
male
female
(f)
pre-pubescent
(g)
% LTP
pre-pub
adult

Figure 1.

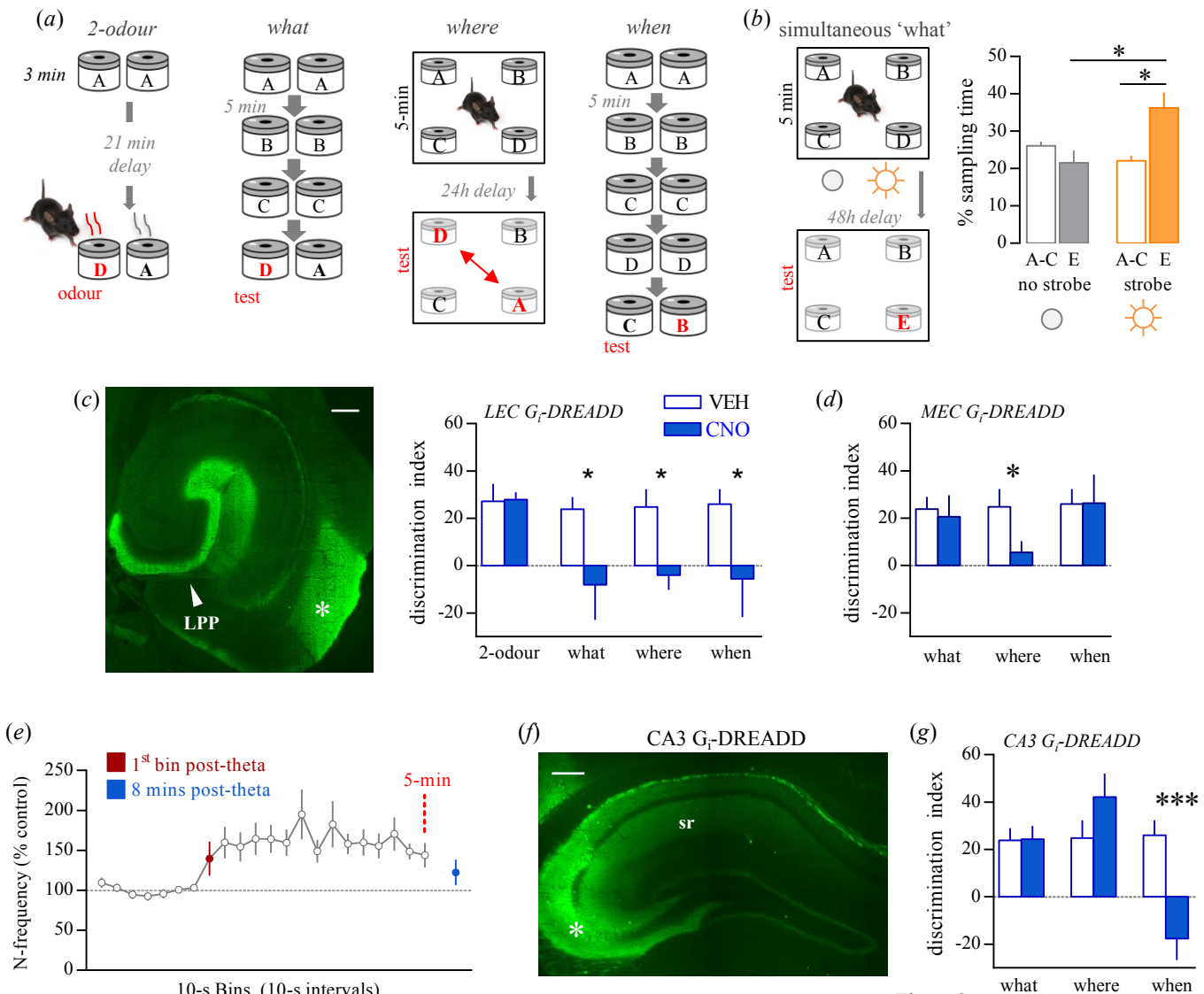
(a)
2-odour
what
where
when
3 min
21 min delay
odour
5 min
test
5-min
24h delay
test
5 min
test
(b)
simultaneous 'what'
5 min
48h delay
test
% sampling time
A-C E
no strobe
A-C E
strobe
(c)
LPP
LEC Gi-DREADD
VEH
CNO
discrimination index
2-odour
what
where
when
(d)
MEC Gi-DREADD
discrimination index
what
where
when
(e)
1st bin post-theta
8 mins post-theta
5-min
N-frequency (% control)
10-s Bins (10-s intervals)
(f)
CA3 Gi-DREADD
sr
(g)
CA3 Gi-DREADD
discrimination index
what
where
when

Figure 2.

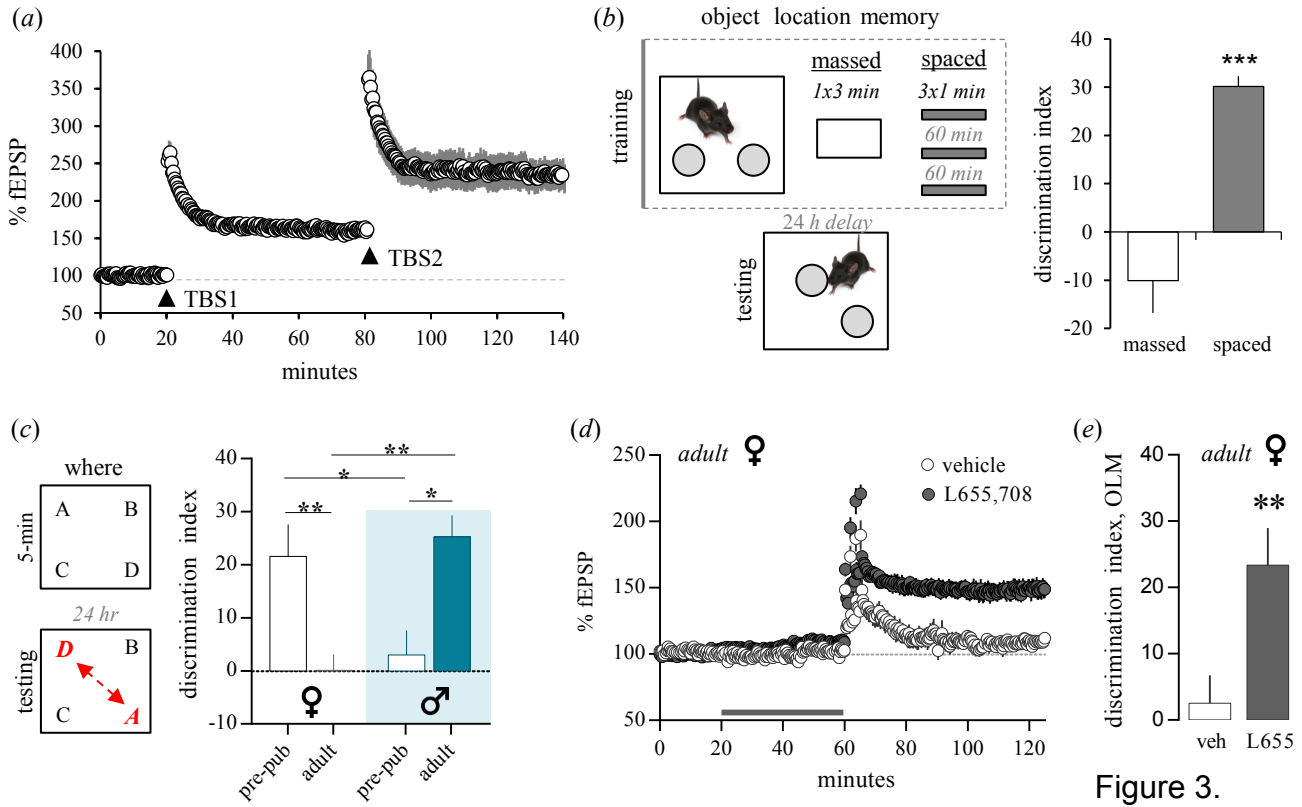
(a)
% fEPSP
minutes
TBS1
TBS2
(b)
object location memory
training
massed
1x3 min
spaced
3x1 min
60 min
60 min
24 h delay
testing
discrimination index
***
massed
spaced
(c)
where
5-min
24 hr
testing
A
B
C
D
discrimination index
pre-pub
adult
pre-pub
adult
(d)
adult
vehicle
L655,708
% fEPSP
minutes
(e)
adult
discrimination index, OLM
**
veh
L655

Figure 3.