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Prepubescent female rodents have enhanced hippocampal LTP and learning relative to males, reversing in adulthood as inhibition increases

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Abstract

Multiple studies indicate that adult male rodents perform better than females on spatial problems and have a lower threshold for long-term potentiation (LTP) of hippocampal, CA3-to-CA1 synapses. We report here that in rodents, prepubescent females rapidly encode spatial information and express low threshold LTP, while age-matched males do not. The loss of low threshold LTP across female puberty was associated with three inter-related changes: increased densities of α5 subunit-containing $GABA_A$Rs at inhibitory synapses, greater shunting of burst responses used to induce LTP, and a reduction of NMDAR-mediated synaptic responses. A negative allosteric modulator of α5-$GABA_A$Rs increased burst responses to a greater degree in adult than in juvenile females and markedly enhanced both LTP and spatial memory in adults. The reasons for the gain of functions with male puberty do not involve these mechanisms. In all, puberty has opposite consequences for plasticity in the two sexes, albeit through different routes.

Keywords

CA1; hippocampus; spatial learning; episodic memory; GABA receptors; long-term potentiation

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Introduction.

There is considerable evidence for sex differences in learning: Among adults, men generally score higher on spatial problems whereas women are frequently better on semantic tests[1,2]. Although for some tasks, differences have been shown to reflect sex-specific learning strategies and influences including task familiarity, the mode of testing, and task demands[3–8], the disparity in spatial learning has been reported for diverse species of mammals suggesting it may be a characteristic feature of the class[9]. Relatedly, sex differences in forms of synaptic plasticity underlying learning have been described. In hippocampal field CA1, Long-Term Potentiation (LTP) is dependent on locally synthesized estrogen in females but not in males[10]. The rate limiting enzyme (p450 aromatase) for synthesis of estradiol (E2), the most prevalent and potent estrogen in brain, is present at high levels in hippocampus and localized to axon terminals[11–13], and E2 levels are several-fold higher in hippocampus than in blood in both sexes[14,15]. However, blocking E2 synthesis disrupts LTP only in females[10,16]. Moreover, at hippocampal CA3-CA1 synapses released estrogen acts through estrogen receptor α (ERα) to activate the LTP critical kinases ERK1/2 and Src, and BDNF receptor TrkB, in females but not males[16]. It thus appears that females require locally produced estrogen to induce critical synaptic modifications whereas males do not. Parallel studies found that the minimum afferent stimulation needed to elicit LTP (the LTP threshold) is greater in adult females than males[16], an effect that is plausibly related to the addition of an estrogen-dependent step in the sequence for inducing potentiation. Given the central role of hippocampus in spatial learning, the higher female LTP threshold helps explain why in rodents adult females require more training to learn object location than age-matched males[16].

The present studies tested if the elevated LTP and learning thresholds identified in female rodents are products of late development. Puberty is a critical landmark in brain maturation and results in a wide array of sex differences in behavior[17–19] but little is known about how it affects the substrates for memory encoding. Past studies showed that in male rats the magnitude of LTP reaches adult levels by the end of the fourth postnatal week and thus in advance of puberty[20–22]. However, these studies did not assess LTP threshold, which is more closely related to neuronal activity occurring during behavior. Studies addressing developmental changes in LTP in female rodents are rare[23], and none have compared the sexes. Thus, it is possible that the sex differences described above are absent or different in prepubertal animals. Such sexually differentiated adjustments during puberty are known to occur in certain psychological domains; anxiety attacks and depression, which occur more frequently in post-pubescent girls than boys, are prominent examples[24–26]. Results described here indicate that the transition from pre- to post-pubertal life causes strikingly different and indeed opposite male vs. female changes in LTP and associated spatial learning. They also describe synaptic mechanisms related to the marked developmental changes that occur in females.

Results.

Hippocampal LTP threshold before vs. after puberty:

We used theta burst stimulation (TBS) to induce potentiation of Schaffer-Commissural (S-C) afferents to field CA1 in acute hippocampal slices from prepubescent (postnatal day (P) 21–28) and young adult (2–3 month old) rats. The amplitudes of field excitatory postsynaptic potentials (fEPSPs) elicited in the CA1 apical dendrites by single pulse stimulation of S-C projections were tightly related to the amplitudes of the preceding fiber volley. Input/output (fiber volley vs. fEPSP amplitude) curves were similar between ages and sex (Fig. 1a). Prior studies using multiple short trains of three bursts, a protocol that is near threshold for eliciting stable potentiation[27], identified a marked sex difference in S-C LTP threshold[16]. We compared effects of four such triplets, applied at 90-sec intervals, on pre- vs. post-pubertal slices. LTP was obtained in adult males but not females (Fig. 1b); the percent potentiation at 55–60 minutes post-induction was 35.0±6.1% and 8.1±4.4%, respectively (P=0.0038, unpaired t-test). The sex difference was also present in short-term potentiation (STP) recorded during the 90 seconds after each triplet ($F_{3,33}$=7.40, P=0.0006, R-M ANOVA; Fig. 1c). After the last triplet, potentiation steadily decayed to baseline in females but not in males (Fig. 1d).

Results for the prepubescent groups were dramatically different than those for adults: theta burst triplets induced robust LTP in females (29.2±4.6% at 55–60 min post-induction) but not in males (3.9±2.7%; unpaired t-test P=0.0005) (Fig. 1e). Sex differences were also evident for STP ($F_{3,42}$=4.181, P=0.01, R-M ANOVA; Fig. 1f). Potentiation was sustained over the subsequent 40 min in females whereas the smaller effect in males quickly fell to baseline (Fig. 1g). In all, theta bursts produced a more pronounced initial potentiation in prepubescent females than in age-matched males and is reflected in the magnitude and stability of subsequent LTP.

Results above indicate that, with age, S-C LTP threshold changes in opposite directions for males vs. females. The difference in percent potentiation at 55–60 min post-induction for pre- vs. post-puberty groups was highly significant for both sexes (2-way ANOVA: $F_{1,25}$=36.35, P<0.0001; Tukey's post-hoc: female: p=0.0015; male: p=0.0004) (Fig. 1h). Burst triplets produced substantially greater STP before than after puberty in females (R-M ANOVA: $F_{3,48}$=11.57, P<0.0001) (Fig. 1i, **right**). In contrast, STP in males tended to be smaller before than after puberty but this was not statistically significant ($F_{3,33}$=1.78, P=0.17; Fig. 1i, **left**). These results raise the possibility that the brief postsynaptic depolarization produced by theta bursts decreases with female puberty resulting in an increase in the number of bursts needed to produce LTP and that male changes across puberty are likely to involve other types of mechanisms.

Spatial learning thresholds before vs. after puberty:

The LTP threshold data (above) lead to the striking prediction that the male-female differences in minimum cue exposure required for spatial memory in young adults would be reversed prior to puberty. We tested this using threshold duration training in the Object

Location Memory (OLM) task (Fig. 2a), a paradigm in which memory is CA1-dependent and facilitated by E2[28–30].

Prepubescent (P25) and adult (2–3 months) male and female mice were given an initial 5-minute exposure to two identical objects ("training" session) and, 24 hours later, a 5-minute "testing" session during which they re-explored the arena with one object displaced towards the center. Despite lacking high levels of circulating estrogen[31], prepubescent females preferentially explored the novel-location object during testing, as indicated by high Discrimination Index (DI) scores, whereas adult females trained during low-estrogen states ("non-proestrus") showed no preference (low DI scores) (Fig. 2b). The opposite developmental pattern held for males: adults performed significantly better than pre-pubertal mice. When comparing sexes in age-matched mice ($F_{1,56}$=27.07, P<0.0001, Interaction 2-way ANOVA), adult males outperformed adult females (P=0.003, Tukey's post-hoc), whereas prepubescent females scored better than prepubescent males (P=0.002). As predicted from the DI scores, prepubescent females and adult males both explored the novel-location object more than the familiar-location object (paired t-tests: P=0.0001 for females, P=0.0006 for males), whereas adult, non-proestrus females and prepubescent males did not (P=0.21, P=0.73, respectively; Extended Data Fig. 1a). As described[16], adult females trained during the high-estrogen stage (proestrus) showed a marked preference for the novel-location object (P=0.004; Extended Data Fig. 1b).

In contrast to the above, adult females given 10 (as opposed to 5) minutes of training discriminated the novel-location object during testing (P=0.01), indicating that they require longer training than prepubescent females to encode spatial memory. Surprisingly, prepubescent males given extended (10-min) training still did not discriminate the moved object (P=0.46, Extended Data Fig. 1b).

Next we tested if the lower facility for rapid spatial learning in prepubescent males and adult females involve problems relating to initial acquisition of cue locations as opposed to later consolidation of the information. Mice were given 5-minute training session and tested for OLM 15-minutes later (Fig. 2a). The DI for non-proestrus adult females was near zero, indicating that they failed to develop short-term memory for object location. In contrast, although prepubescent males did not encode long-term memory for cue location as assessed at 24h, they had excellent DIs after the short delay (adult female vs prepubescent male; P=0.0002; unpaired t-test) (Fig. 2c; Extended Data Fig. 1c). These data reinforce the conclusion from the STP analyses in the physiological experiments that the reversal of sex differences across puberty is due to different types of changes in female vs. male plasticity.

Total cue sampling times (both objects) during OLM training and testing were greater for adult than prepubescent mice (females: P≤0.0002, males: P≤0.0012) but did not differ between the sexes at either age (Supplementary Fig. 1a,b). Thus, the absence of long-term memory in adult females was not due to a failure to investigate the objects and their locations. Inattention could however have been a factor in the poor performance by prepubescent males (but see below). There were no evident differences between groups in locomotor activity in the behavioral sessions (Supplementary Fig. 1c).

The above findings raise the question of whether differences in spatial learning are specific to simple tasks such as OLM or instead occur in the same subgroups in more complex circumstances. We tested this using a paradigm in which mice freely sampled four distinct and equally salient odors for 5 minutes and were tested for preferences 24 hours later with positions of two of the odors swapped ('Where' task; Fig. 2d). Note that the animals sequentially investigate a collection of cues under these circumstances and that recognition of changes requires encoding of both cue location and identity. Thus the task is considerably more challenging than OLM and incorporates both 'what' and 'where' features of episodic memory. The animals sampled the four odors to similar degree during training with no evident differences between groups (Extended Data Fig. 1d). The DI indicated that adult, non-proestrus females did not discriminate the novel-location cues at testing whereas prepubescent females spent significantly more time with moved odors (Fig. 2e) ($p=0.008$, paired t-test, Extended Data Fig. 1e). Males exhibited the opposite pattern: prepubescent males had a DI near zero whereas adults preferentially attended to the moved cues (Fig. 2e). A two-way ANOVA ($F_{1,34}=21.38$, $P<0.0001$) of the four groups showed that the adult females and prepubescent males performed worse than the other two groups ($P\leq0.043$, Tukey's post-hoc).

In contrast to the OLM results, there were no age or sex differences in the total time spent investigating the four odors during training or testing for the episodic 'where' task (Supplementary Fig. 1d,e). Thus, in this case, the absence of long-term memory in post-pubescent females and prepubescent males cannot be attributed to different sampling times. There were no evident differences in locomotion during behavioral sessions (Supplementary Fig. 1f).

We then tested additional groups to determine if there are sex or age differences in a version of the multi-cue task with a minimal spatial component (episodic "What" test). Mice were allowed to sample four odors for 5 minutes and were tested 24 hours later with one of the cues replaced by a novel odor (Fig. 2d; Extended Data Fig. 1f). In contrast to results for the combined cue identity/location problem, all four groups showed a clear preference for the novel odor at testing (Fig. 2f; Extended Data Fig. 1g). Total sampling times were similar to those for OLM in that adult males and females sampled the cues longer than their prepubescent counterparts (Supplementary Fig. 1h), but this did not appear to influence retention scores. Locomotor activity was similar between groups (Supplementary Fig. 1i). These findings establish that sex and age do not significantly affect encoding cue identity, but that changes across puberty produce opposite effects on female vs. male learning when a spatial component is introduced.

Factors relating to the developmental changes in female LTP:

The STP results described above could reflect age- and sex-differences in the depolarizing responses produced by theta burst triplets and thus the likelihood of triggering the initial steps leading to LTP. Comparisons of the composite response to a single four-pulse theta burst in rat slices confirmed that the amplitudes of the 2^{nd} through 4^{th} fEPSP were substantially larger in females before vs. after puberty ($F_{3,90}=24.40$, $p=5.38\times10^{-12}$; RM-ANOVA) (Fig. 3a). These results are suggestive for underlying mechanisms. Afferents

from CA3 monosynaptically innervate both CA1 pyramidal cells and local interneurons, some of which form inhibitory synapses in the same dendritic field (stratum radiatum) as the excitatory contacts[32]. Inhibitory postsynaptic currents (IPSCs) are slower than the fast AMPAR-mediated glutamatergic currents and thus exert their greatest shunting effects on EPSCs triggered by the 3rd and 4th pulses in a theta burst[33, 34].

We tested the possibility of an age-related increase in feedforward IPSCs in females using voltage-clamp recordings to compare effects of the $GABA_AR$ antagonist bicuculline on the size of a burst response in prepubescent and adult rat slices (Fig. 3b). In agreement with the field recordings, the area of the baseline (pre-drug) burst response, normalized to the amplitude of the first EPSC in the burst, was nearly twice as large in pre-pubertal females (60.6±2.8 pA*msec) as in adult females (31.3±3.6 pA*msec; P=0.0002, unpaired t-test). The burst response area tended to decrease with age in males (pre- vs. post-puberty: 61.4±3.5 vs. 51.0±4.3 pA*msec) but this effect did not reach statistical significance (p=0.068, unpaired t-test; Supplementary Fig. 2a). The percent decrease from prepubescence to adulthood was significantly larger in females (−48.3±5.9%) than in males (−16.9 ±7.1%) (p=0.004, unpaired t-test). Bicuculline had a larger effect after puberty: in females it increased the area of a single burst response by 82.5±22.9% and 195.8±29.7% in prepubescent and adult animals, respectively (P=0.02, unpaired t-test) (Fig. 3c). The antagonist had a measurably greater effect on normalized burst responses in males than females before (P=0.019, unpaired t-test) but not after (P=0.273) puberty. These results indicate that feedforward IPSCs during a burst response increase across puberty in females and thereby more potently shunt the depolarization produced by a burst in adults.

Delivery of two bursts separated by 150–200 msec results in a marked facilitation of the second response due to partial suppression of feedforward IPSPs[35,36]. The enhanced and prolonged depolarization unblocks NMDARs and thus initiates the sequence leading to the production of LTP[37]. We used the selective NMDAR antagonist APV to estimate the magnitude of the NMDAR-component of the burst response before vs. after female puberty. A pair of theta bursts was delivered under control conditions and then again after 30 minutes of APV infusion. As expected from above, the area of the composite potential produced by the first (control) theta burst was substantially larger in slices from prepubertal than adult female rats (P<0.0001) (Fig. 3d,e). Notably, the amplitude of the initial fEPSP within the burst was virtually identical in the two groups (Prepuberty: 2.10±0.02 mV, Adult: 2.11±0.03 mV. P=0.64, unpaired t-test) indicating that the age difference in burst responses was due to the 2nd-4th potentials. The prepubescent cases also had a larger response to the second theta burst than did adults during baseline testing (159.9±4.8 vs. 119.1±4.7 mV*msec respectively, P=0.0003) (Fig. 3e). Following APV infusion, the response to the first of the two theta bursts was not measurably different than that before APV infusion for either age group but the magnitude of the second burst response was reduced in both cases. We subtracted the waveforms of the APV-plus responses from those acquired before APV infusion to quantify the drug effect (Fig. 3f). This analysis confirmed that the antagonist had no effect on the first burst response but removed a significant component of the second, an effect that was clearly greater in pre- vs. post-puberty slices (−26.4±4.6 vs. −12.1±3.8 mV*msec, respectively. P=0.024) (Fig. 3g). This accord with the prediction that

the NMDAR-mediated component of the composite response decreases across puberty in females.

$GABA_AR$ synapses before vs. after female puberty.

The above findings indicate that some element of fast ionotropic GABAergic transmission activated by CA3 inputs to CA1 changes during female puberty. We accordingly counted the number of inhibitory synapses in the apical CA1 dendritic subfield evaluated in the physiological studies. Dual immunofluorescence for the postsynaptic scaffolding protein gephyrin and the presynaptic vesicular GABA transporter (VGAT) was used to label inhibitory synapses in female rats; 3-D reconstructions of several thousand individual pre- and postsynaptic elements were created from image z-stacks and quantified using Fluorescence Deconvolution Tomography[38,39] (FDT) (Fig. 4a). There was no change in the incidence of inhibitory synapses (Fig. 4b) or the per-synapse density of gephyrin (Fig. 4c) or VGAT (not shown) immunoreactivity (-ir) from 4- to 8-weeks of age.

Next, we evaluated specific $GABA_AR$ subunits co-localized with gephyrin beginning with the α5 subunit that has been linked to feedforward inhibition in CA1[40]. Specifically, α5-$GABA_ARs$ are present in inhibitory synapses on CA1 pyramidal cell dendrites where they mediate slow decaying IPSCs. Deletion of α5 decreases the amplitude of spontaneous (synaptic) IPSCs in CA1 and increases paired-pulse facilitation of fEPSPs elicited by S-C stimulation. The latter result constitutes evidence that a significant portion of feedforward inhibition, which shunts the response to the second stimulation pulse, is mediated by α5-$GABA_ARs$.

The α5 subunit levels at inhibitory (gephyrin-ir) synapses in CA1 stratum radiatum increased markedly from 4- to 8-weeks of age in females (Fig. 4d,e); this was evident as a right-shift in the immunolabeling density frequency distribution towards higher values in adults relative to juveniles ($F_{50,700}$=14.46, P<0.0001, RM-ANOVA) (Fig. 4d, left). As expected from this, the percentage of synapses associated with high concentrations of α5-ir was more than two-fold greater after puberty (Fig. 4d, right). This developmental change was not present in males: the frequency distributions for densities of synaptic α5-ir were superimposable for prepubescent and adult groups ($F_{10,130}$=0.46, P=0.91) (Supplementary Fig. 2b) and the percent of contacts with high concentrations of α5-ir were comparable (pre-puberty: 20.6±1.3%; post-puberty: 20.5±2.2%) (Fig. 4d, right). Comparisons of males and females identified an interaction between sex and age ($F_{1,26}$=15.30, P=0.0006, 2-way ANOVA). The density of synaptic α5-ir increased across female puberty (P<0.0001), and pre-pubescent males had higher levels than age-matched females (P=0.033). There were no differences between juvenile and adult males (P=0.99) or between adult males and females (P=0.07). In all, there was a striking difference between the sexes with regard to puberty-related changes in a key element of feedforward GABAergic transmission in the CA1 apical dendritic field.

We analyzed two additional $GABA_AR$ subunits to test the selectivity of the pre- to post puberty increase in α5 recorded for females. From 4- to 8-weeks of age, the density of immunoreactivity for the α2 subunit at gephyrin-ir synapses was unchanged ($F_{50,700}$=0.17,

P>0.99, RM-ANOVA) (Fig. 4f) and there was a slight shift towards higher densities of β1 subunit-ir but this was not statistically significant ($F_{50,700}$=1.32, P=0.07) (Fig. 4g).

Effects of blocking α5-GABA$_A$Rs.

We tested the prediction that blocking α5-GABA$_A$Rs with subunit selective negative allosteric modulators (NAMs, a.k.a., inverse agonists) would restore theta burst responses and LTP in adult female rats to levels found before puberty. Infusion of the selective α5-NAM L655,708 (150 nM, 40 minutes), which acts via the benzodiazepine binding site to suppress IPSCs gated by α5-GABA$_A$Rs, had minimal effects on baseline fEPSP amplitude in adult females but caused a clear enhancement of the normalized and raw amplitudes of the 2nd-4th potentials in the composite response to a theta burst, as expected for suppression of fast inhibition (Fig. 5a for normalized values; raw values: 1st pulse, vehicle: 2.75±0.13 mV, L655,708: 3.00±0.11 mV, two-tailed unpaired t-test p=0.11; RM-ANOVA across pulses: P=0.0048, $F_{3,39}$=5.041). These results constitute the first evidence that α5-GABA$_A$Rs potently affect theta burst responses. We confirmed that theta burst triplets fail to elicit LTP in vehicle-treated adult female rat slices but do produce robust LTP in the presence of L655,708 (9.3±2.9% vs. 48.9±6.7%; P=0.0002, at 55–60 min post-TBS, unpaired t-test) (Fig. 5b). Voltage-clamp recordings in mouse slices revealed that the NAM significantly increased (22.2±4.8%, P=0.003; paired t-test) the area of single theta burst responses above pre-treatment baseline in adult females. However, and in accord with the analysis of α5 densities at synapses, the compound had little if any effect (8.3±4.2%, P=0.144) on response size in prepubescent female slices (prepubertal vs adult; P=0.04, unpaired t-test) (Fig. 5c). Collectively, these findings indicate that the pronounced increase in synaptic α5-GABA$_A$Rs that occurs over the course of female puberty depresses theta burst responses leading to an increase in LTP threshold.

As expected from the FDT analysis of synaptic α5 concentrations, L655,708 caused a comparable increase in burst response area in males before and after puberty (% area change: 26.6±3.4 and 29.6±4.7, respectively; p<0.01 within groups, paired t-tests. p=0.649 between age groups, unpaired t-tests. Supplementary Fig. 2c).

Next, we tested the prediction that the recovery of low threshold LTP in adult females produced by L655,708 would result in a comparable improvement in spatial learning. Non-proestrus mice were injected with vehicle or the α5-NAM 30 minutes before 5-minute OLM training and were tested for discrimination of the moved object 24 hours later (Fig. 2a). Vehicle-treated mice had low retention scores whereas the L655,708 group performed at the level of prepubescent females and adult males (vehicle vs. L655,708: P=0.0097, unpaired t-test) (Fig. 5d; Extended Data Fig. 2a). The NAM did not influence cue sampling times during training or testing, or locomotor activity (Extended Data Figs 2b,c,d).

Contributions of ERα to LTP prior to puberty.

As described, LTP in adult females is dependent on locally synthesized estrogen acting on synaptic ERα[16]. We investigated the possibility that this requirement emerges with puberty, and associated increases in circulating estrogen[31], and thus is a potential contributor to the elevation of LTP thresholds. The selective ERα antagonist MPP produced a near complete

blockade of potentiation induced by a train of 10 theta bursts in slices from 4-week old female rats (Vehicle: 58.9±7.2% at 55–60 min post-TBS; MPP: 12.4±4.5%; P=0.0006, unpaired t-test) (Fig. 6a). The use of a stronger induction paradigm (10 bursts instead of burst triplets) emphasizes the extent to which prepubescent female LTP is dependent on this estrogen receptor class. Using FDT we determined that ERα levels at PSD-95-ir synapses in CA1 stratum radiatum are dramatically higher in adult than in prepubertal female rats ($F_{19,342}$=22.10, P<0.0001) (Fig. 6b). This result argues against a decline in ERα contributing to the age-related increase in LTP threshold. We previously showed that synaptic ERα levels are substantially higher in adult females than age-matched males[16], but this sex difference was not detected in prepubescent rats (Supplementary Fig. 2d,e). Moreover, synaptic concentrations of ERβ-ir in prepubertal and adult females were comparable (Fig. 6c). Together, these findings raise the possibility that the marked and sex-specific change in synaptic ERα levels is a specialization that partially compensates for the reduction in the NMDAR-component of the theta burst response that occurs in female rodents during the transition through puberty.

Discussion.

The present results lead to the surprising conclusion that the polarity of sex differences at hippocampal synapses and related learning reverses from before to after puberty. This occurs because of opposite developmental changes in females vs. males: thresholds for plasticity and encoding spatial information increase in females and decrease in males. We identified a plausible mechanism for the female effect: the depolarizing responses elicited by the short high-frequency stimulation bursts are substantially larger in the prepubertal animals. The bursts produce frequency facilitation of transmitter (glutamate) release at the S-C synapses but the expected enhancement of successive postsynaptic responses is partially shunted by the buildup of di-synaptic feedforward IPSCs. The latter GABAergic responses are due to interneurons engaged by CA3-CA1 projections. Shunting is of considerable functional significance with regard to LTP because enhanced and prolonged depolarization is required to unblock the voltage-dependent, relatively slow NMDARs that initiate the complex sequences leading to synaptic modifications. We found that the shape of the composite response elicited by a four-pulse burst differed significantly between pre- vs. post-pubescent females: responses to later pulses in the burst were larger in prepubertal animals suggesting less inhibition at this age. Clamp recordings demonstrated that feedforward inhibition during the bursts increases dramatically at some point during female puberty.

The facilitation of responses that occurs when two bursts are given sequentially was also greater before than after female puberty. Previous work showed that the enhancement of the second response reflects a partial refractoriness of feedforward inhibition due to $GABA_BR$ activation on interneuron terminals[41,42]. These metabotropic receptors open potassium channels and thereby reduce GABA release probability with the effect maximized at about the period of the theta wave. We interpret the greater facilitation of the second burst in prepubescent females as resulting from the refractory process operating on weaker inhibition. This effect is directly related to LTP threshold because the greater and temporally extended depolarization produced by the second burst suffices to open NMDAR channels.

We confirmed that the NMDAR-component of the second response is larger before than after puberty in females.

Despite age-related changes in activity-driven IPSCs, we did not detect an increase in the number or density of GABAergic contacts in CA1 stratum radiatum. This finding raised the possibility of developmental changes in the composition of $GABA_A$Rs. The pentameric GABA receptors include one gamma, two alpha, and two beta subunits[43]. The specific α subunits exert differential effects on rate kinetics. Receptors containing α5 are of particular interest in the present context because they generate large and prolonged IPSCs that shunt NMDAR-mediated currents in the apical dendrites of field CA1[44]. Notably, adult hippocampus in rodents and humans has unusually high levels of α5 expression[45–47]. Our analyses indicate that α5 levels at inhibitory synapses in apical field CA1 are substantially lower before puberty in females. The developmental difference in α5-$GABA_A$Rs in the same dendritic layer containing the excitatory S-C synapses that generate the theta burst response helps explain why later potentials in those responses are unusually large in prepubescent females. In accord with the above arguments, we found that negative modulation of α5-$GABA_A$Rs restored theta burst responses, LTP thresholds, and spatial learning in adult females to levels found before puberty.

The changes in α5 concentrations could reflect the developmental onset of the estrous cycle, which occurs around P28–30 depending on rodent strain and species[31], as multiple studies have shown this influences the $GABA_A$R subunit composition[48–51]. These effects have been related to fluctuations in progesterone and its neurosteroid metabolites[49]. The steroids operate over different time courses and mechanisms including effects on $GABA_A$R subunit gene expression[50,51]. These analyses have only recently extended to α5 in rodents[52] and as yet do not provide an interpretation for the present findings. An alternative possibility involves the late maturation of interneurons and their connections. Neuronal activity affects expression of cell-specific transcription factors, including Npas4, that influence the formation of excitatory synapses on somatostatin-positive interneurons[53,54]. Relatedly, network activity influences elements of perineuronal nets associated with parvalbumin-positive interneurons, a specialization widely held to alter synaptic connectivity[53,55]. There is also evidence that activity influences the expression of channels by parvalbumin interneurons, including the potassium and voltage-gated Kv1.1, that regulate the excitability and firing characteristics of these cells[56,57]. It is thus possible that the change from pre- to post-pubertal life is associated with changes in interneuron function that alter postsynaptic cells in a manner that shifts the balance of inhibitory synapses in favor of those enriched with α5-$GABA_A$Rs.

The absence of age-related changes in the density of α5-ir at inhibitory synapses in males constitutes one of the more striking sex differences observed in the present studies. It is clear from this, and related observations, that changes in shunting IPSCs and theta burst responses are not responsible for the emergence of low threshold LTP in post-pubertal males. In males, burst responses were if anything reduced from before to after puberty which strongly suggests that NMDAR-gated ionic currents did not increase. There remains the possibility that calcium influx through the receptors, or the subsequent release of the cation from intracellular stores, increases from 4- to 8-weeks of age in males. Alternatively, recent

studies raise the possibility that NMDAR-mediated activation of the LTP critical kinase Src involves non-ionic functions; a metabotropic route is also suggested for NMDAR-dependent ERK activation[58,59]. A change in the linkages between the NMDARs and these enzymes during puberty could account for the observed drop in male LTP threshold.

Given the extensive evidence linking LTP to some but not all forms of learning, an increase in the threshold for inducing LTP is likely to have significant consequences for behavior. In accord with this, post-pubescent females did not acquire simple or complex spatial information with a minimal number of trials, tests on which excellent scores were observed prior to puberty. Importantly, pharmacological suppression of α5-$GABA_A$Rs restored LTP and memory encoding in adult females to levels observed prior to puberty. One interpretation of the seemingly deleterious elevation of plasticity and learning thresholds is that the effects are secondary to adaptations for other, unrelated female behaviors. The α5-containing $GABA_A$Rs have been linked to anxiety[60–62], a psychological variable that can be strongly affected in a sex-specific manner by puberty[63–65]. Possibly, then, an adaptation involving emotional behaviors appropriate to the transition to early adult life affects learning mechanisms as a side effect. Moreover, slower encoding could have advantages in complex real world environments that contain multiple cues and choices, circumstances in which it is necessary to distinguish reliable signals from noise. If so, then adaptive pressures relating to typical mammalian sex differences in the variety and extent of social roles, including extremely complex care of altricial offspring, may have resulted in opposing late developmental adjustments to learning mechanisms.

Finally, evidence that hippocampal LTP threshold changes in opposite directions, between males and females, in the transition to post-pubertal life raises the question of whether there are similar effects in other brain regions. This issue has yet to be addressed but there is reason to expect the hippocampal changes influence broader network function. The hippocampus works in concert with parahippocampal and medial prefrontal cortex[66], and cooperativity with the latter is reportedly critical for realizing estrogen effects on encoding spatial and episodic memory[67,68]. This suggests that hippocampal changes described here likely influence functions of the larger hippocampal-prefrontal cortical system, including behaviors ascribed to the cortical field.

Materials and Methods.

Animals.

The studies used postnatal day (P) 21–26 and 2–4 month old male and female mice (FVB129 background) and P21–28 and 2–3 month old Sprague Dawley male and female rats. We used rats for most of the electrophysiological (excepting the use of mice for analysis in Fig. 5c) and all of the immunolabeling experiments because their larger hippocampus allowed for greater precision in aligning zones used in the imaging methods with those sampled during recording studies. Mice were used in all behavior experiments to allow for larger sample size and because tasks have been validated in mice. Animals were on a 12 hr light/dark cycle with lights on at 6:30AM and food and water *ad libitum*. Mice were grouped by 3–5 littermates and rats by 2–4 littermates per cage in rooms maintained at 68°F and 55% humidity. Experiments were conducted in accord with NIH guidelines

for the Care and Use of Laboratory Animals and protocols approved by the Institutional Animal Care and Use Committee at University of California, Irvine. All adult females were estrous staged using vaginal lavage as described[69, 70] and identified as proestrus (presence of nucleated cells) and non-proestrus (cornified cells for estrus stage and leukocytes for metestrus/diestrus). Prepubescent females were defined as P21–26 in mice and P21-P28 in rats to reflect differences on puberty onset, as defined visually by vaginal canal opening in both species[31]. Prepubescent male rats and mice were age-matched to conspecific females.

Object Location Memory (a.k.a., object placement).

Behavioral experiments were performed as described[16, 71] in FVB129 mice. Animals were handled for two minutes per day for two days and then habituated to an empty arena (30×25cm floor, 21.5 cm walls) for 5 min on each of the subsequent 4 days. For training, mice were returned to the arena containing two identical glass funnels that were 1 cm away from two adjacent corners, were allowed to explore for 5 or 10 minutes, and then were returned to their home cage. After a specified time delay, the mice were placed in the same arena with one funnel displaced towards the center and allowed to explore for 5 minutes.

Adult female mice were monitored for estrous cycling for at least 7 days prior to the training day to ensure normal cycling. On the training day animals were separated into proestrus vs non-proestrus (estrus, metaestrus, diestrus were pooled) groups[12]. Prepubescent mice were P25 at training and did not show evidence of vaginal opening on training or testing days. For studies with L655,708, adult female mice were randomly assigned to the drug or vehicle group on the training day.

For these and other behavioral paradigms, the animal's movements within the test chamber were recorded by overhead camera during both training and testing. Videos were hand-scored by individuals blind to group, and sampling time was recorded when the animal's nose sniffed the object within 0.5cm. Sampling was not noted if the animal's nose was in the same zone while turning their head but not attending to the object. A discrimination index (DI) was calculated by $100*(t_{\text{novel object}} - t_{\text{familiar object}})/(t_{\text{novel object}} + t_{\text{familiar object}})$.

Episodic "Where" Task.

Studies used FVB129 mice as previously described[72]. The mice were handled one day prior to the task. On training day, the mice were then placed into a plexiglas arena (60×60 cm floor, 30 cm walls) containing four empty glass cups (5.25 cm diameter x 5 cm height) with a metal lid with a single hole (~1. 5 cm diameter). The cups were removed from the chamber, and the animal rested undisturbed for 5 minutes. For the training session, the four cups were re-introduced with each containing one of the following odorants dissolved in mineral oil (final concentration of 0.1 Pascals): (A) (+)-Limonene (≥97% purity, Sigma-Aldrich), (B) Cyclohexyl ethyl acetate (≥97%, International Flavors & Fragrances Inc.), (C) (+)-Citronellal (~96% Alfa Aesar), and (D) Octyl Aldehyde (~99%, Acros Organics). The animal's behavior was monitored over 5 minutes of odor exposure, and then they were returned to their home cage for 24 hours. For testing, the animals were allowed to explore the chamber with either odorant pairs (A:D) or (B:C) switched in position for 5 minutes.

Episodic “What” Task.

The task followed the same set up as in the episodic “Where” task, but, for training, the animals were exposed to odorant B, C, D, and E (Anisole; ~99%, Acros Organics) for 5 minutes. The next day, the animals returned to the chamber with odorant A replacing odorants B or D and were allowed to explore the arena for 5 minutes.

For Episodic Where and What tasks, video recording of behavior were hand-scored by observers that were blind to odors and groups. Cue sampling time was designated as the time the animal’s nose was oriented towards the odor hole and within a 0.5 cm radius. The DI for the “Where” task was calculated as follows: $100*(t_{\text{SUM OF SWITCHED PAIR}}-t_{\text{SUM OF STATIONARY PAIR}})/(t_{\text{TOTAL SAMPLING}})$. For the “What” task, DI was: $100*(t_{\text{NOVEL}}-t_{\text{MEAN FAMILIARS}})/(t_{\text{TOTAL SAMPLING}})$.

Extracellular Hippocampal Slice Recording.

Transverse rat hippocampal slices (370μm thick) were prepared using the McIllwain chopper and then transferred to an interface recording chamber, with constant oxygenated artificial cerebrospinal fluid (aCSF) perfusion (60–70mL/hr; 31±1°C) as described[16]. The aCSF was composed of (in mM): 124 NaCl, 26 $NaHCO_3$, 3 KCl, 1.25 KH_2PO_4, 2.5 $CaCl_2$, 1.5 $MgSO_4$ and 10 dextrose (pH 7.4, 300–310 mOsm). Recordings started 1.5–2 hours after slice preparation. Field EPSPs were elicited using a twisted nichrome wire stimulating electrode and recorded with glass pipette electrode (2M NaCl, R=2–3MΩ). These electrodes were placed in dorsal CA1b stratum radiatum equidistant from the CA1 stratum pyramidale. Single pulse baseline stimulation was applied at 0.05Hz with baseline intensity set to 50–60% of the maximum population-spike free fEPSP amplitude. All recordings were digitized at 20kHz using an AC amplifier (A-M Systems, Model 1700) and sweeps of 1.5 seconds duration were recorded every 20 seconds using NAC 2.0 Neurodata Acquisition System (Theta Burst Corp. Irvine, CA). After baseline recording and additional drug infusion, LTP was induced by applying 10 bursts of TBS (4 bursts at 100 Hz, 200 ms interval between bursts) or threshold TBS (4 triplets of theta bursts with 200 ms interval between bursts within the triplet and 90 seconds between triplets), and the recording to baseline (0.05Hz) stimulation resumed for 1 hour. Female rat vaginal smears for estrous monitoring were collected post-mortem at time of slice preparation, and only non-proestrus animals were included in LTP experiments. Experiments using APV included all stages as there were no statistical differences between proestrus and non-proestrus stages. For all electrophysiology experiments, multiple slices from at least 4 rats were used, and the N reported was number of slices.

Whole-Cell Voltage Current Clamp Recording.

Hippocampal slices were prepared on the horizontal plane at a thickness of 350 μm from 4- and 8-week-old mice and rats using a Leica vibrating tissue slicer (Model: VT1000S). Slices were placed in a submerged recording chamber and continuously perfused at 2 mL/min with oxygenated (95% O_2/5% CO_2) aCSF at 32°C. Whole-cell recordings (Axopatch 200A amplifier: Molecular Devices) were made with 4–7 MΩ recording pipettes filled with a solution containing (in mM): 140 $CsMeSO_3$, 8 CsCl, 10 HEPES, 0.2 EGTA, 2 QX-314, 2 Mg-ATP, 0.3 Na-GTP. Osmolarity was adjusted to 290–295 mOsm and pH 7.4.

Bipolar stimulating electrodes were placed in CA1 stratum radiatum, 100–150 μm from the recording cell. EPSCs were recorded by clamping the pyramidal cell at −50 mV in the presence of 50 μM APV. Data was collected using Clampex 10.6 and analyzed with Clampfit 10.6 (Molecular Devices).

Drug Application.

For hippocampal slice recording, the compounds were infused into the aCSF bath via an independent perfusion line (6mL/hr). Final aCSF bath concentrations: APV (100 μM; Hello Bio #HB0225), bicuculline (20 μM; Tocris Bioscience #0130), L655,708 (field electrophysiology: 150 nM, whole-cell electrophysiology: 50 nM; Tocris Bioscience #1327), MPP dihydrochloride (3 μM; Tocris Bioscience #1991) were dissolved in DMSO (<0.01%). For the Object Location Memory task, vehicle (0.1% DMSO) or L655,708 (0.5mg/kg in 0.1% DMSO) was injected intraperitoneally 30 min prior to training.

Fluorescence Deconvolution Tomography (FDT).

Prepubescent female and male rats were sacrificed on P25; all adult female rats were sacrificed during the diestrus stage of the estrous cycle (P57–59), and adult male rats were age-matched. Slide-mounted, fresh frozen tissue sections (25 μm; coronal) from mid-septotemporal hippocampus (same region used for electrophysiological studies) were immersion-fixed in 4% paraformaldehyde and processed for dual immunofluorescence as previously described[38, 39, 71, 73] with incubation in primary antisera at 4°C for 24 hours and in secondary antisera at room temperature for 2 hours. After the secondary incubation, sections were washed in 0.1M phosphate buffer and cover-slipped using VectaShield with DAPI (Vector Labs).

Primary antisera cocktails included rabbit anti-gephyrin (1:2000, Abcam #ab32206; RRID: AB_1860490) combined with either guinea pig anti-β1-$GABA_AR$ (1:500, Synaptic Systems #224705; RRID: AB_2619940) or mouse anti-α2-$GABA_AR$ (1:500, Abcam #ab193311; RRID: AB_2890213); mouse anti-gephyrin (1:1500, Synaptic Systems #147021; RRID: AB_2232546) combined with either guinea pig anti-vGAT (1:2000; Synaptic Systems #131004; RRID: AB_887873) or rabbit anti-α5-$GABA_AR$ (1:800, Abcam #ab10098; RRID: AB_296840); rabbit anti-ERα (1:700; Santa Cruz Biotechnology; #sc-542; RRID: AB_631470) combined with mouse anti-PSD95 (1:1000, Thermo Fisher #MA1–045; RRID: AB_325399); and mouse anti-ERβ (1:700, Santa Cruz Biotechnology #sc-390243; RRID: AB_2728765) combined with goat anti-PSD-95 (1:1000; Abcam #ab12093; RRID: AB_298846). Secondary antibodies (all at 1:1000 dilution) included donkey anti-rabbit Alexa Fluor 594 (Invitrogen, Thermo Fisher Scientific #A-21207; RRID: AB_141637), goat anti-guinea pig Alexa Fluor 488 (Invitrogen, Thermo Fisher Scientific #A-11073; RRID: AB_2534117), donkey anti-goat Alexa Fluor 488 (Invitrogen, Thermo Fisher Scientific #A-11055; RRID: AB_2534102), and donkey anti-mouse Alexa Fluor 488 (Invitrogen, Thermo Fisher Scientific # A-21202; RRID: AB_141607).

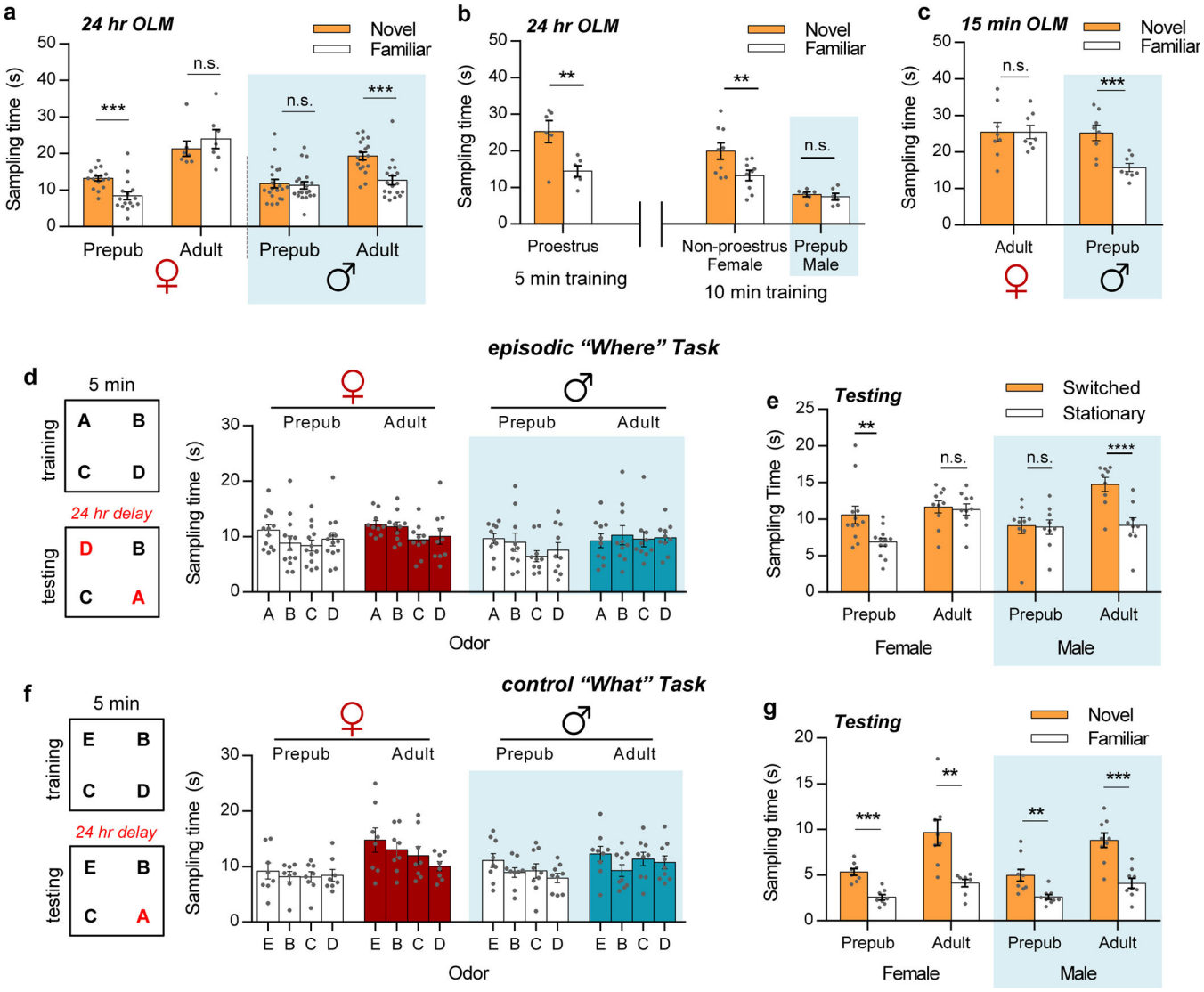
FDT was conducted as described[16, 38, 71, 73]. Under 63x magnification image z-stacks (136×105×2 μm) were collected using 200 nm steps from CA1 stratum radiatum; for each case images were collected from ≥5 sections spaced by 250 μm on the septotemporal axis

of hippocampus. The images were processed for iterative deconvolution (99% confidence, Volocity 4.0). Individual stacks were used to construct 3-D montages of each sample field. Labeled objects were detected using threshold image segmentation across each channel separately; an image was normalized and thresholded at a given intensity threshold, erosion and dilation were used to fill holes and remove background pixels, and objects were segmented based on connected pixels above a threshold using in-house software (using C99, Java (openJDK IcedTea6.1.12.6), Matlab R2019b, PuTTY 0.74, and Perl v5.30.0). All immunofluorescent elements meeting size constraints of synapses, and detected across multiple intensity thresholds, were quantified using automated systems. The gephyrin and PSD-95-immunoreactive elements were considered to be double-labeled for the second antigen if there was contact or overlap in fields of the two fluorophores as assessed in 3-D. Using this approach, ~30,000 inhibitory or excitatory synapses were reconstructed per sample field, and >150,000 were analyzed per rat.

Statistics.

Results are presented as mean±SEM. For LTP studies significance was determined by comparing the within-slice normalized mean response over the last 5 minutes of the experiment between groups using 2-tailed unpaired t-test or two-way ANOVA with post-hoc Tukey test. Significance in STP and burst pulse analyses was determined using repeated-measure (RM) ANOVA with Bonferroni post-hoc test. Whole-cell recordings and input-output curves were analyzed using 2-tailed unpaired and paired t-test and linear regression, respectively. Behavioral data was analyzed with two-way ANOVA with post-hoc test Tukey for age and sex comparisons, or unpaired or paired 2-tailed Student’s t-test for two group comparisons. For FDT density frequency distributions, significance was determined using 2-way RM-ANOVA; for statistical comparison of mean data between two groups a Student’s t-test was used, and for age and sex comparisons a two-way ANOVA was used followed by post-hoc Tukey test. All analyses were performed using Prism 6.0 (GraphPad).

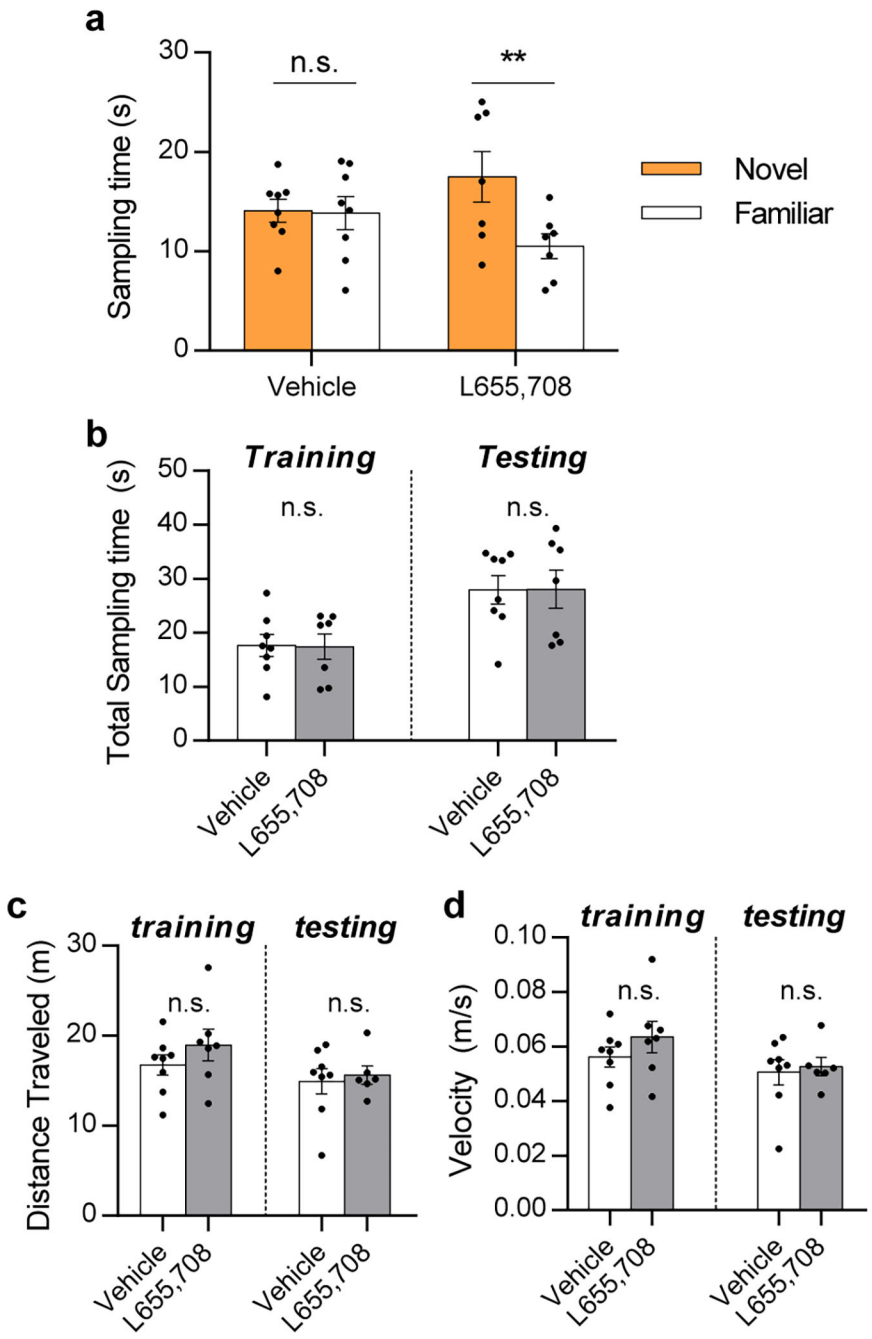
Extended Data



Extended Data Fig. 1. Sampling times for each cue in Object Location Memory (OLM), "Where", and "What" tasks across age and sex.

(**a**) OLM (5-min training session, tested 24 hours later): Sampling times of displaced (Novel) vs the stationary (Familiar) objects were compared for male and female mice of prepubescent (Prepub) and adult ages. Prepubescent females and adult males preferentially sampled the displaced object (***P=0.0001, ***P=0.0006, respectively; 2-tailed paired t-test), whereas non-proestrus adult females and prepubescent males did not (n.s. P=0.21, P=0.73, respectively; N=7–18/group). (**b**) OLM (5- or 10-min training, tested 24-hours later): Adult females trained for 5 minutes during proestrus stage preferentially sampled the displaced object over the stationary object (**P=0.004). Non-proestrus adult females and prepubescent males were trained for 10 minutes. Non-proestrus females preferred the displaced object (**P=0.01), but the prepubescent males did not (n.s. P=0.46; N=6–9/group). (**c**) OLM (5-min training, 15-min delay): Adult females did not spend more

time with the moved object (P=0.99), whereas Prepub males preferred the moved object (***P=0.0002; N=8/group). (**d**) *Left.* Schematic for episodic "Where" task with four odors (see Methods). *Right.* Sampling times of odors A-D for each group during the 5-minute training trial (One-way ANOVA: P>0.05 within all age groups). (**e**) Sampling times for the "switched" pair (Novel) vs the stationary pair. Prepubescent females and adult males sampled the "switched" pair more than the stationary pair (2-tailed paired t-test: Prepub female **P=0.008, Adult male ****P=0.00004). Prepubescent male and adult females showed no preference (P=0.65, P=0.97, respectfully; N=8–11). (f) *Left.* Schematic of the "What" task (see Methods). *Right.* Sampling times for each odor (One-way ANOVA: P>0.05 within all age groups; N=8–9/group). (**g**) Sampling times for novel odor vs mean of the three familiar odors (2-tailed paired t-test: ***P<0.001, **P<0.01; N=8–9/group). Data are represented as mean±SEM. Detailed statistics are found in Supplemental Table 1.



Extended Data Fig. 2. Exploration data for 24-hour delay Object Location Memory in adult, non-proestrus female mice given L655,708.

(**a**) Sampling times for displaced (Novel) vs stationary (Familiar) object for Vehicle (2-tailed paired t-test: n.s. P=0.85) and L655,708 (**P=0.009). (**b**) Total sampling times for training and testing were comparable for treated vs. vehicle groups (2-tailed unpaired t-test: n.s. training P=0.95, testing P=0.98). (**c**) Distance traveled was comparable (2-tailed unpaired t-test: training P=0.29, testing P=0.71) and (**d**) velocity was similar between treatments (training P=0.30, testing P=0.73). For all panels, Vehicle N=8, L655,708 N=7. Data presented as mean±SEM. Further statistics found in Supplemental Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement.

The data that support the findings of this study are available in this manuscript and supplementary information.

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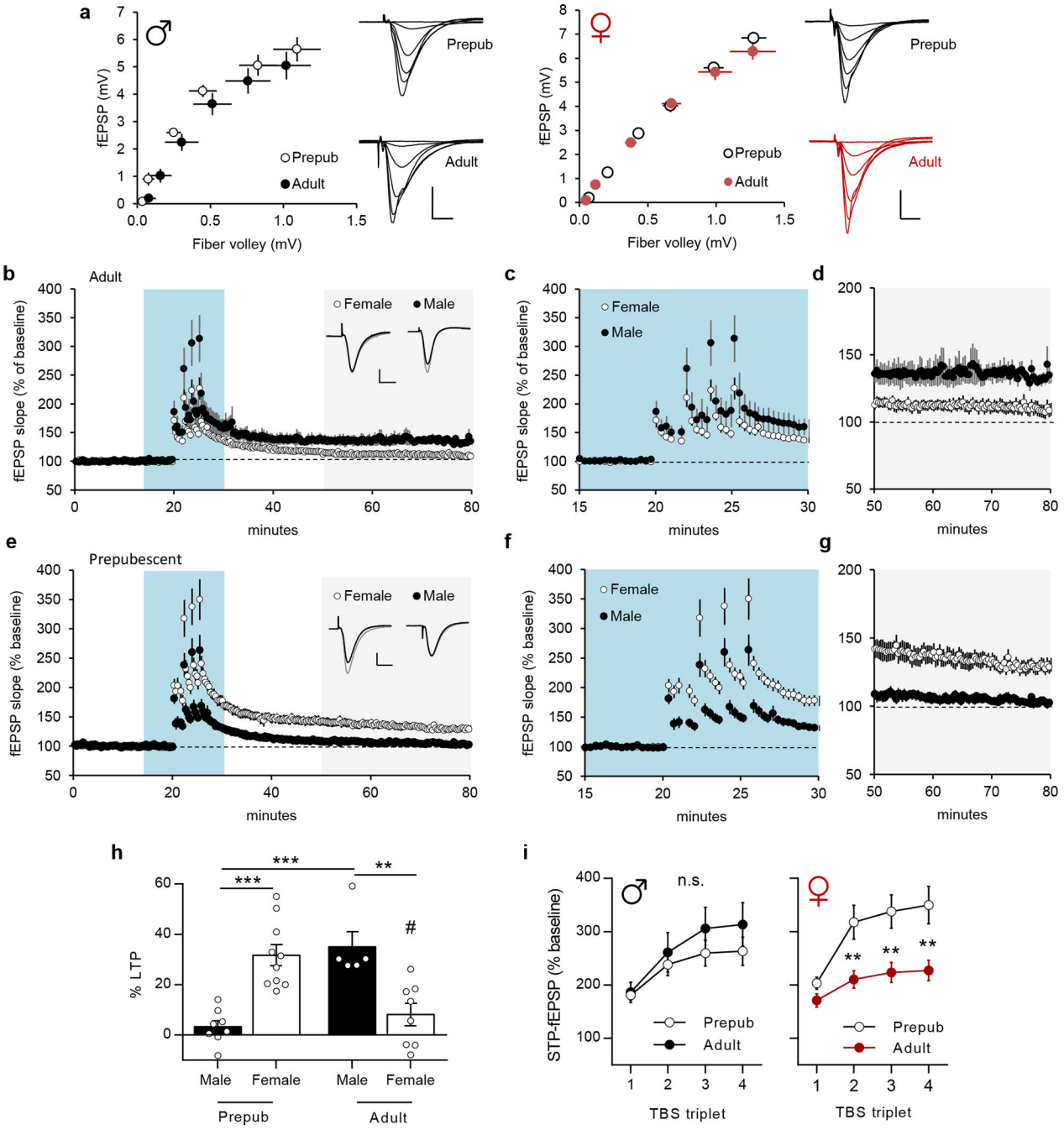

Figure 1. Sex differences in adult rat LTP thresholds are reversed before puberty.

fEPSPs elicited by stimulation of the Schaffer-commissural (S-C) projections were recorded in CA1 stratum radiatum in acute hippocampal slices from prepubertal ('Prepub'; 3–4 week) and adult (8–10 week) rats of both sexes. (**a**) Input-output curves were comparable across age and sex (P=0.46, $F_{3,124}$=0.86, linear regression). Representative traces on right. Bars: 2mV, 5ms. (**b**) Four theta burst 'triplets', spaced by 90 seconds, were delivered to S-C axons after 20 minutes of baseline. Each triplet included three bursts (1 burst: 4 pulses at 100Hz) separated by 200ms. After the triplets, single pulse responses in adult females decayed

steadily to baseline whereas those in males fell to a stable plateau just above baseline values. Inset: superimposed traces from baseline (black) and the end of the recording session (gray). Bars: 1mV, 10ms. (**c**) Stretched x-axis (minutes 15–30 of panel 'b') to illustrate single pulse responses during the 90 seconds after each triplet; male fEPSPs were elevated above those of females, indicating a sex difference in short-term potentiation (STP). (**d**) Stretched axes (minutes 50–80 of 'b') show that adult males had a lasting increase in fEPSPs after TBS whereas females did not. (**e-g**) Same format as that for panels b-d but for prepubescent rats: Responses recorded for 90 seconds after each triplet are much larger in females than males (**f**), the reverse of the pattern recorded in adults, and females expressed robust LTP whereas males did not (**g**). (**h**) Summary of LTP (55–60 minutes) for both age groups and sexes (Two-way ANOVA Interaction: $F_{1,25}$=36.35, P=0.00005; Tukey Post-hoc: ***P<0.001, **P<0.01, #P=0.0015 for adult female vs prepub female). (**i**) Averaged fEPSP slope responses (percent baseline) collected following each of the four theta burst triplets. There was no reliable difference for STP in prepubescent vs. adult male slices (2-way RM-ANOVA: $F_{3,33}$=1.781, P=0.17), but STP was larger in prepubescent female vs. adult female slices ($F_{3,42}$=11.57, P=8.44×10^{-6}; Bonferroni post-hoc: **P<0.01). For all panels, N=5–10. Mean±SEM values shown. Statistics summarized in Supplementary Table 1. n.s., not significant.

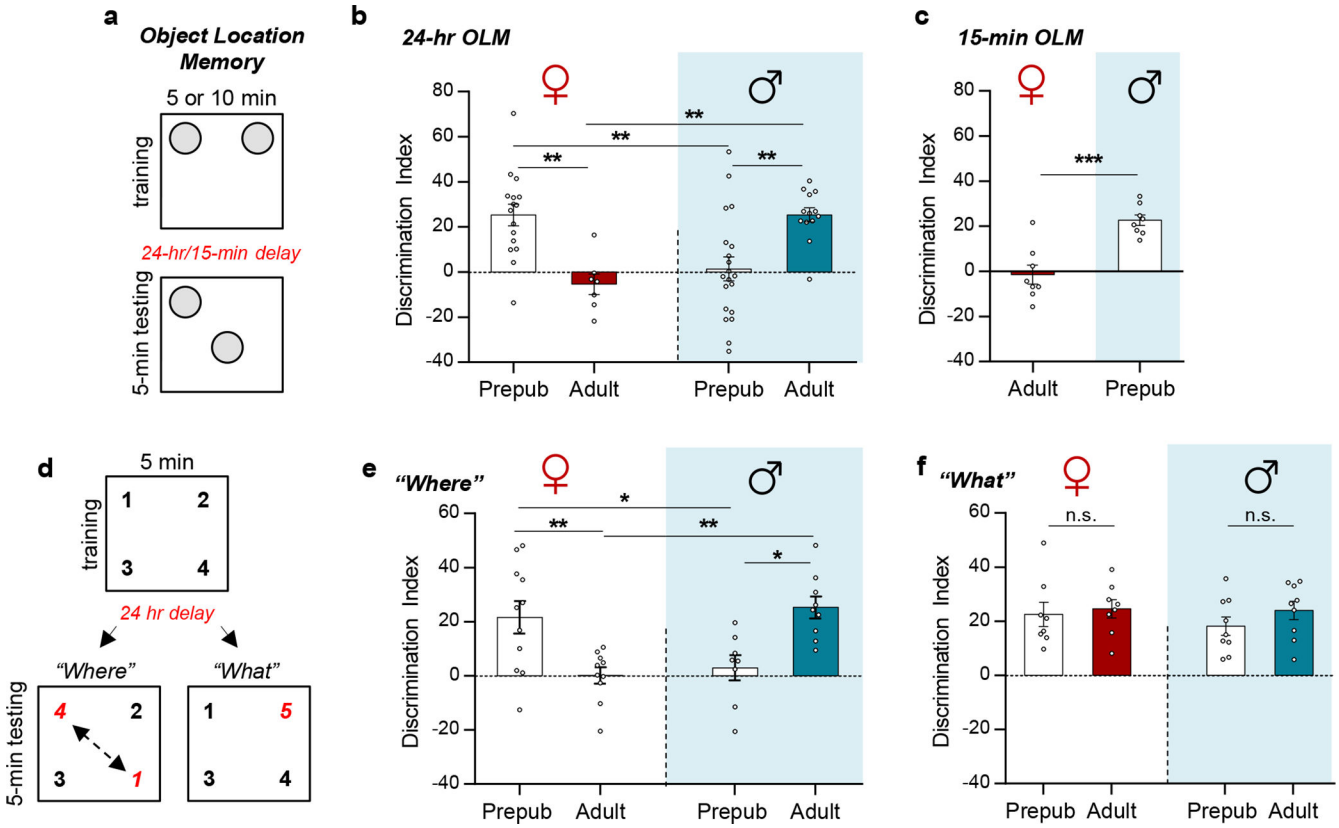

Figure 2. Adult sex differences in spatial learning are reversed prior to puberty.

(**a**) Object Location Memory (OLM) paradigm. Mice were exposed to two identical objects for 5 minutes ('training'). After a delay, mice were returned to the chamber for 5 minutes with one object displaced ('testing'). (**b**) With a 24 hour delay, prepubescent ("Prepub") females (N=16) discriminated the displaced object, whereas adult females (N=7) (2-way ANOVA (Interaction of sex and age): $F_{1,56}$=27.07, P=2.9×10^{-6}; Tukey's post-hoc: **P=0.0034) and prepubescent males (N=20) (**P=0.002) did not. Conversely, adult males (N=17) better discriminated the moved object than prepubescent males (**P=0.0016) and adult female mice (**P=0.003). (**c**) When tested 15 minutes post-training, adult females performed significantly worse in the task than prepubescent males (2-tailed unpaired t-test: ***P=0.0002, N=8/group). (**d**) Episodic "Where" and "What" task schematic. Mice were exposed to four different odors for 5 minutes (training); 24 hours later, the animals explored the chamber with either two objects switched in position ("Where") or one odor replaced by a novel odor ("What"). (**e**) In the "Where" task, prepubescent females better discriminated the switched odor pairs than did adult females ($F_{1,34}$=21.38, P=5.25×10^{-5}. post-hoc: **P=0.0091) and prepubescent males (*P=0.043). Adult males better discriminated switched odors compared to prepubescent males (*P=0.016) and adult females (**P=0.003) (N=8–11/group). (**f**) In the "What" task, all groups preferentially explored the novel odor vs the familiar odors at testing ($F_{1,30}$=0.26, P=0.61. N=8–9/group). Mean±SEM values shown. Statistics summarized in Supplementary Table 1.

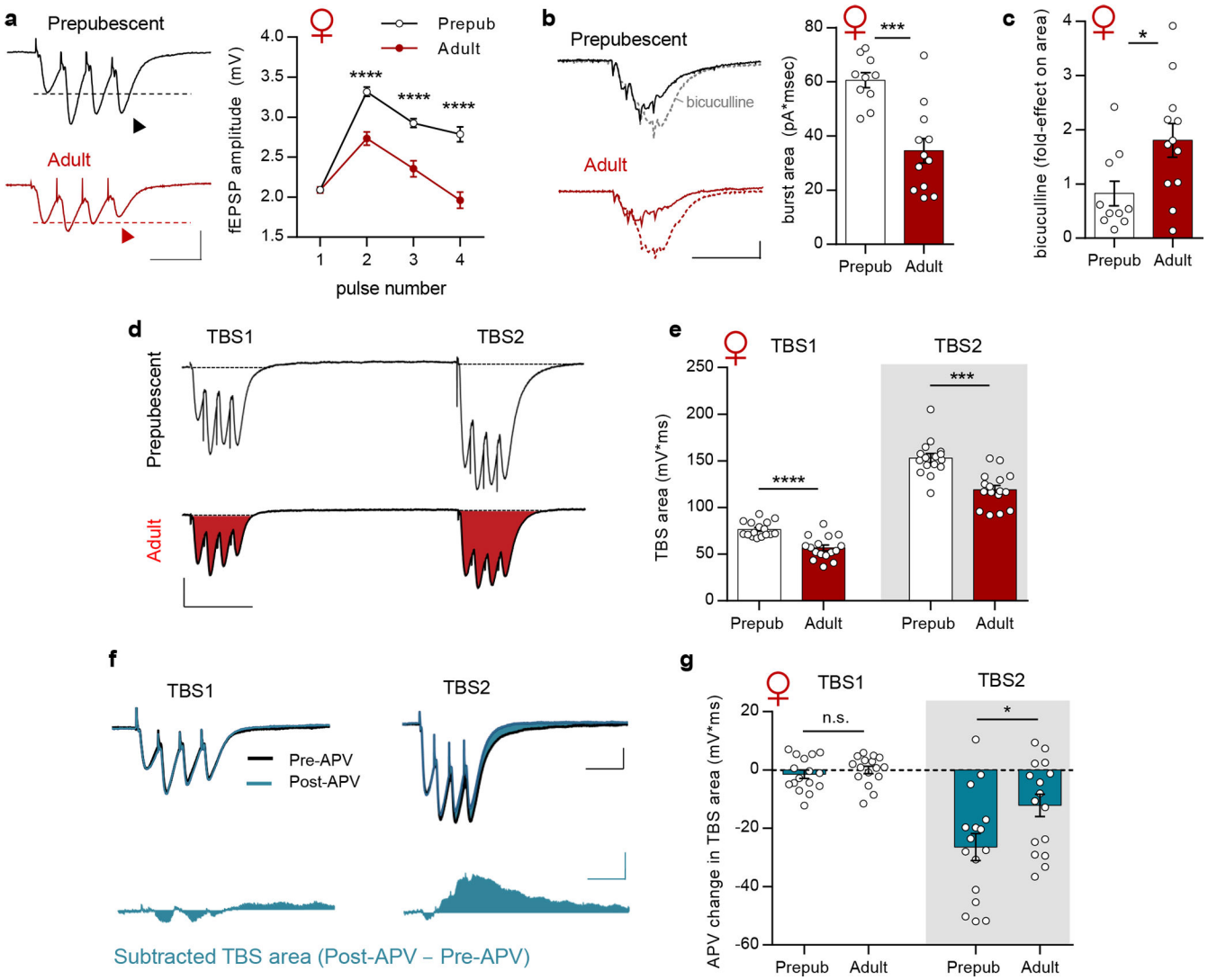

Figure 3. Theta burst responses, feedforward inhibition, and NMDAR-mediated synaptic potentials differ between pre- vs. post-pubescent female rats.

(**a**) Traces of fEPSP responses elicited by a single burst (four pulses,100Hz) delivered to the Schaffer-commissural (S-C) projections and recorded from CA1 stratum radiatum in slices from prepubescent ('Prepub') or adult female rats. Arrowheads mark the fourth fEPSP in the burst response. Bars: 2mV, 20msec. *Right*: Graph shows the four fEPSPs within the burst responses in adult and prepubescent groups (2-way RM-ANOVA: $F_{3,90}$=25.40, P=5.38×10^{-12}. Asterisks indicate group differences for each fEPSP in the burst response: Bonferroni post-hoc, ****P<0.0001). (**b**) Voltage-clamp recordings from CA1 pyramidal cells during delivery of single burst stimulation to S-C projections in slices from a prepubescent or adult female rats before (solid trace) and during (dotted) infusion of bicuculline (20 μM, 10 min). In adults the baseline response is smaller and the $GABA_AR$ antagonist had a greater effect (bars: 100 pA, 50 ms). Graph shows areas of baseline (pre-drug) burst responses for prepubescent (N=10) and adult (N=12) groups: the size of the composite four EPSC responses (normalized to amplitude of the first response) were nearly twice as large in prepubescent vs. adult females (2-tailed unpaired t-test: ***P=0.0002).

(**c**) Group data for effects of bicuculline before vs. after puberty. The antagonist had substantially larger effect in slices from the older rats (2-tailed unpaired t-test: *P=0.02). (**d**) Traces show S-C responses elicited by two theta bursts (4 pulses at 100 Hz, 200 ms interval) in prepubertal and adult female slices (bars: 1 mV, 50 ms). (**e**) Group data for areas of responses elicited by the first and second theta bursts. As in 'a', the first of these (TBS1) was larger in prepubertal as compared to adult female slices (2-tailed unpaired t-test: **** $P=7.4\times10^{-6}$); this was also the case for the composite response to the second burst (TBS2) (***P=0.0003). (**f**) Traces show superimposed theta burst responses before and after 30-min infusion of NMDAR antagonist APV (100μM) (bars: 1 mV, 25 ms). The bottom graphs (solid blue) show the results of subtracting the baseline responses to TBS1 and TBS2 from those recorded in the presence of APV (bars: 0.5 mV, 25 ms). Note that APV reduced the later segments of the negative-going response to TBS2 while having minimal effects on that produced by TBS1. (**g**) Summary of group data for effects of APV on the size of responses to two theta bursts: APV did not alter the composite response to TBS1 in prepubescent or adult slices (n.s. P=0.46) but reduced the areas of responses to TBS2. The attenuation of the TBS2 response was larger prior to puberty (*P=0.024, 2-tailed unpaired t-test). For panels (**a, e, g**): N=16/group. Data are presented as mean values +/− SEM.

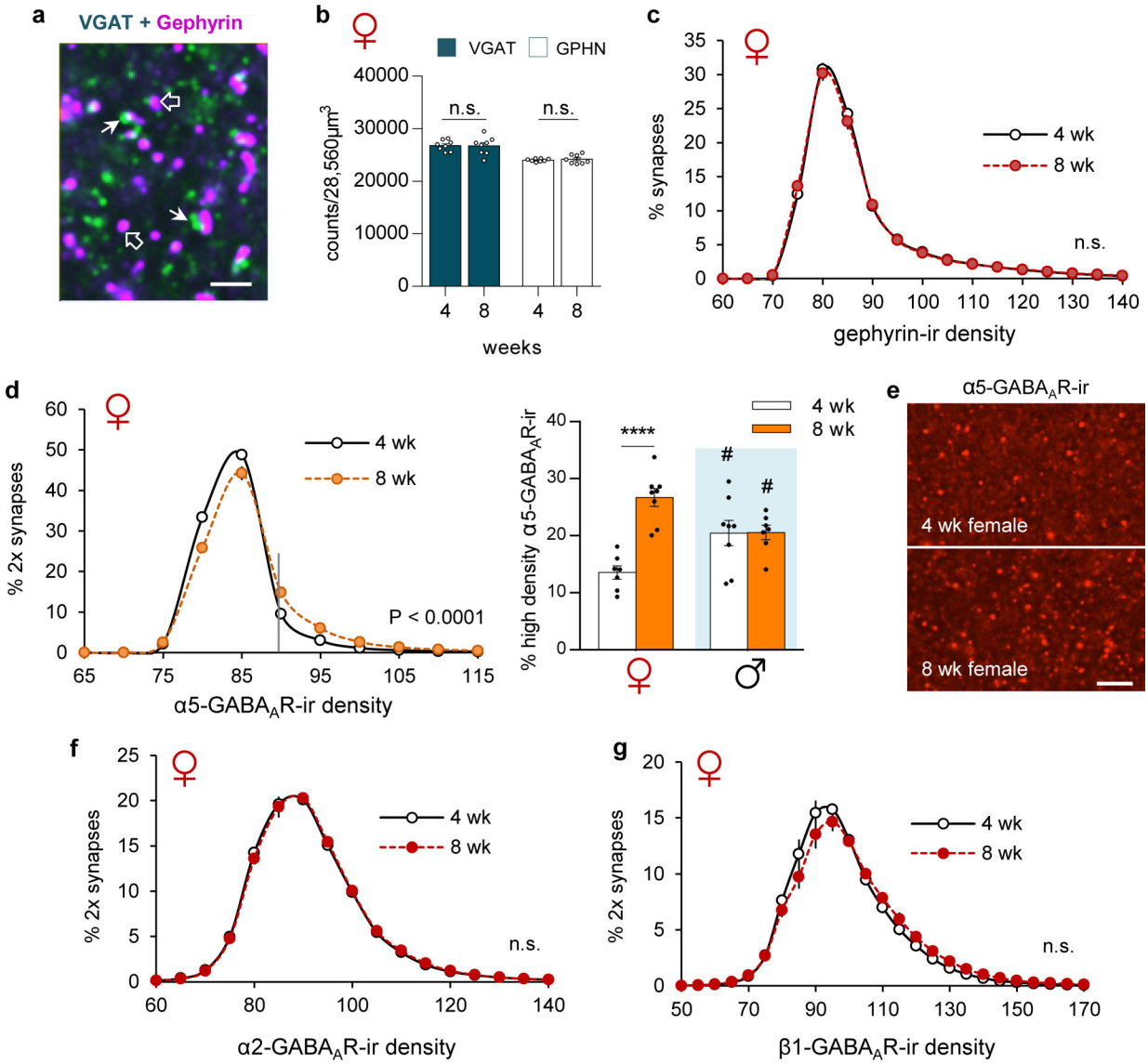

Figure 4. Synaptic levels of GABA$_A$R subunits in pre- vs. post-pubescent female rat.

(**a-c**) Dual immunofluorescence for presynaptic vesicular GABA transporter (VGAT) and postsynaptic gephyrin (GPHN) in CA1 stratum radiatum indicates that numbers of inhibitory synapses are equivalent in the two age groups. (**a**) Image shows immunofluorescence localization of gephyrin (open arrows, magenta) and VGAT (closed arrows, green) in an 8-week old female. Calibration bar: 2 μm. (**b**) Counts of VGAT-immunoreactive (-ir) and GPHN-ir puncta (mean±SEM) were comparable at 4- and 8-weeks of age (2-tailed, unpaired t-test: VGAT, P=0.92; GPHN, P=0.78). (**c**) Immunolabeling density frequency distributions for gephyrin-ir elements did not differ between groups ($F_{40,560}$=0.58, P=0.98). (**d-e**) The density of immunolabeling for the α5-GABA$_A$R-ir at inhibitory synapses changes from 4 to 8 weeks in females, but not in males. (**d**) *Left.* Plot shows the density frequency distributions for α5-ir co-localized with gephyrin ('2x synapses') in females: the curve for the 8-week group was markedly right-shifted towards higher densities relative to that for the 4-week group (P=5.8×10^{-78}, $F_{50,700}$=14.46). *Right.* Graph shows the percentage

of double-labeled synapses with dense α5-ir (above vertical cutoff line on the frequency distribution curve) in female and male rats (Supplementary Fig. 2b for males frequency distribution): Values were significantly higher in post- vs pre-pubescent females, whereas males showed no change between ages (2-way ANOVA Interaction $F_{1,26}$=15.30, P=0.0006; Tukey's post-hoc: ****P<0.0001, #P<0.05 vs. 4 wk female. P=0.99 for 4wk vs 8wk male). (**e**) Photomicrographs of α5-$GABA_A$R-ir in CA1 stratum radiatum illustrate the increase in densely-immunolabeled puncta in 8- vs 4 -week old females. Bar: 5 μm. (**f,g**) Density frequency distributions for (**f**) α2-$GABA_A$R-ir and (**g**) β1-$GABA_A$R-ir show that levels of synaptic immunoreactivity (colocalized with gephyrin) did not differ between pre- and post-pubescent females (α2: P>0.99, $F_{50,700}$=0.17; β1: P=0.07, $F_{50,700}$=1.32). For all panels N=7–8/group. Statistics on (**c-d, f-g**) performed with 2-way RM-ANOVA (Interaction). Data are presented as mean values +/− SEM. n.s., not significant.

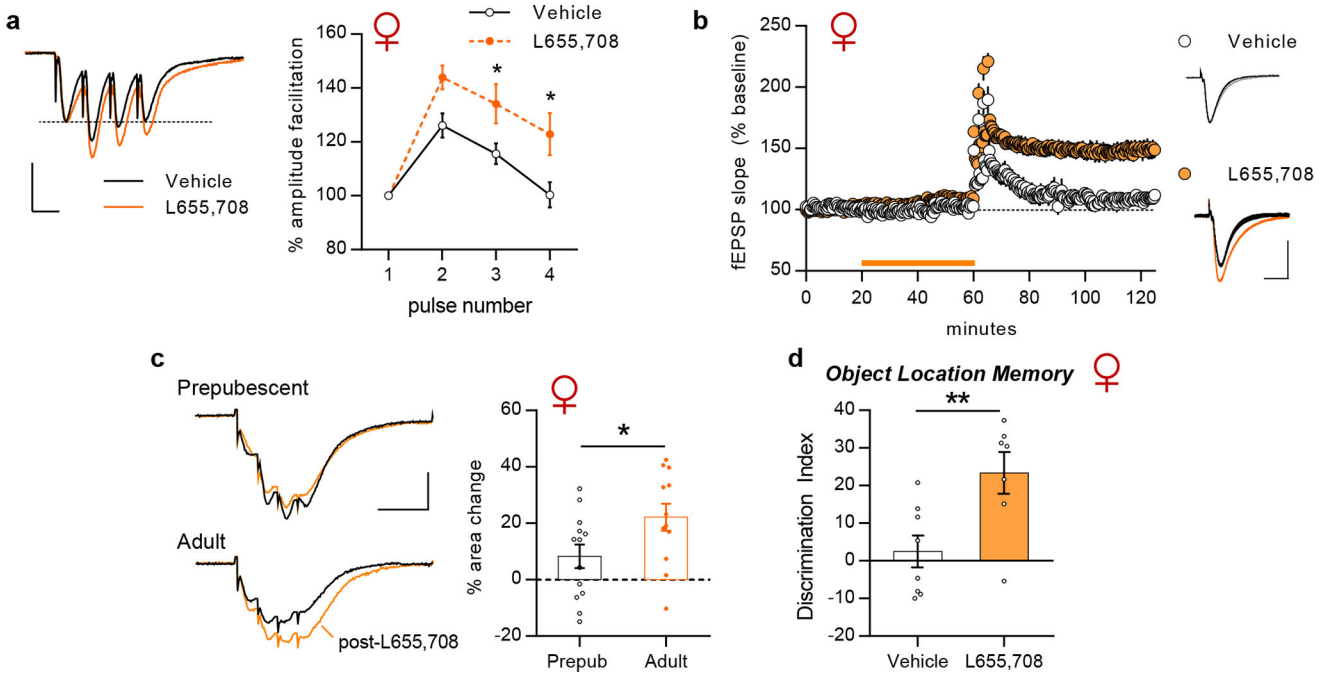

Figure 5. A negative allosteric modulator (L655,708) of the α5-GABA$_A$R subunit increases theta burst responses and facilitates LTP in adult females.

(**a,b**) Baseline responses were collected for 20 min before a 40-min infusion of L655,708 (150 nM) in adult female rat hippocampal slices. Four TBS 'triplets' (90 sec intervals) were applied to induce LTP after drug treatment. (**a**) *Left.* L655,708 increased the size of the response to one theta burst (4 pulses, 100 Hz) relative to vehicle-treatment (bars: 2mV, 10ms). *Right.* Group data (N=7–8/group) for the four fEPSP amplitudes normalized to the first pulse that comprise a single theta burst response (2-way RM-ANOVA: P=0.011, $F_{3,39}$=4.21; Bonferroni post-hoc (3rd pulse: *P=0.048 and 4th pulse: *P=0.012). (**b**) Female slices treated with L655,708 (line bar) express robust LTP whereas those infused with vehicle do not (2-tailed unpaired t-test: P=0.0002 at 1 hour post-TBS. N=7/group). Right: Representative superimposed traces. Black: baseline, Orange: post-LTP, bars: 2 mV, 10 ms. (**c**) Voltage-clamp recordings from CA1 neurons elicited by a single theta burst from prepubescent vs adult female mouse slices before (black trace) and during infusion with L655,708 (50 nM, 10 min; bars: 50 pA, 25 ms). Bar graph summarizes results for prepubescent and adult female slices: L655,708 significantly increased the area of the burst response in adult (N=12; P=0.003, 2-tailed, paired t-test) but not prepubescent females (N=13; P=0.144). The difference in % increase above pre-drug baseline for the two groups was significant (2-tailed, unpaired t-test: *P=0.04). (**d**) Adult female mice were intraperitoneally injected with vehicle (0.1% DMSO) or L655,708 (0.5mg/kg) 30 minutes before the 5-minute training trial for Object Location Memory. With a 24 hour delay, adult females given L655,708 showed enhanced discrimination for the displaced object compared to vehicle-treated females (2-tailed unpaired t-test: **P=0.0097. Vehicle N=8, L655,708 N=7). Mean±SEM values shown. Statistics summarized in Supplementary Table 1.

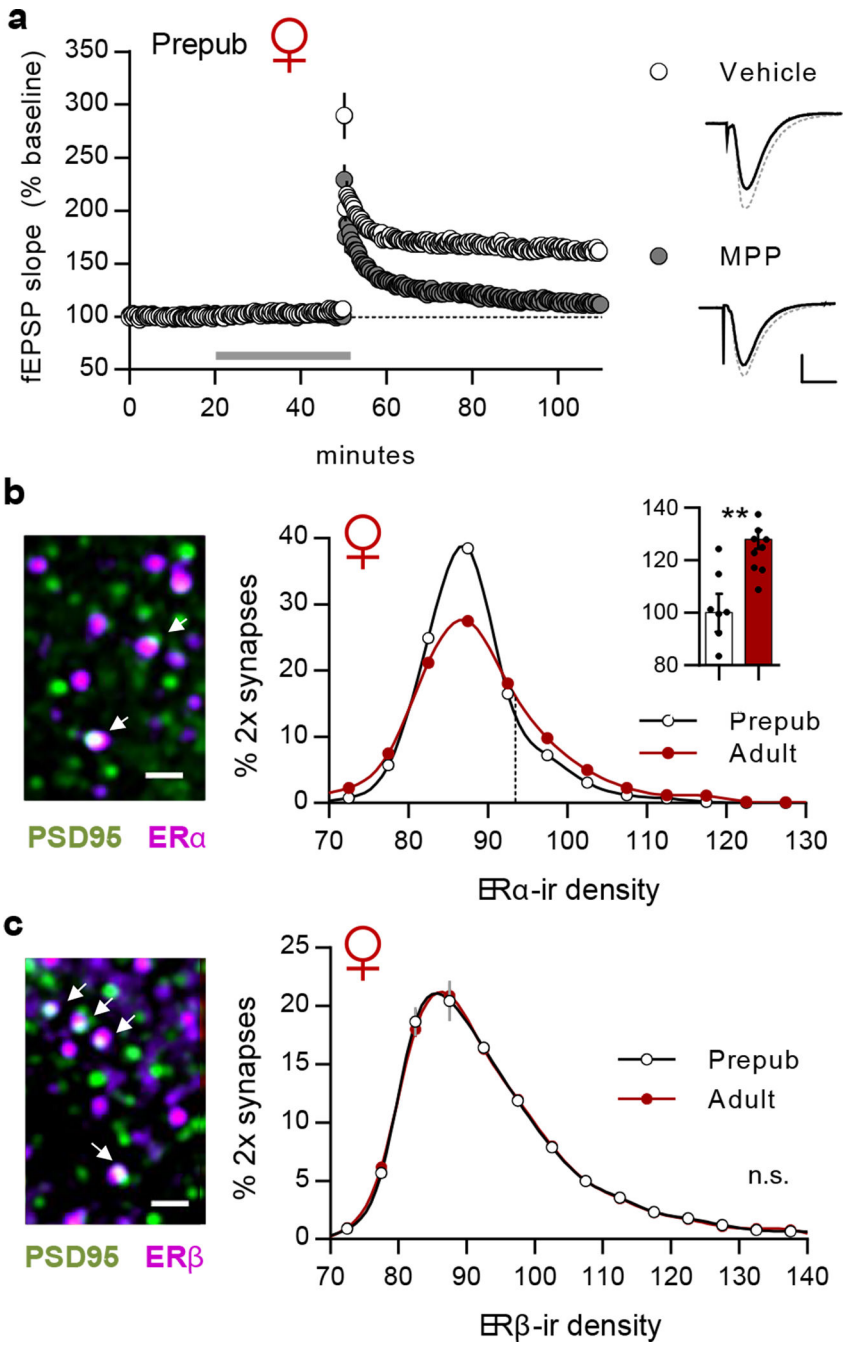

Figure 6. LTP induction in prepubescent female rats is dependent on activation of estrogen receptor α (ERα).

(**a**) Slices from prepubertal female rats were infused for 30 minutes with ERα antagonist MPP or vehicle before S-C stimulation with 10 theta bursts. In the presence of MPP, LTP decayed to baseline within an hour, while vehicle-treated cases exhibited robust and stable LTP (2-tailed unpaired t-test at 55–60 min post-TBS: P=0.0006; N=5/group). Traces (right) show superimposed pre- (black) vs. post-TBS (dashed) fEPSPs. Bars: 1 mV, 10 ms. (**b**) Dual immunolabeling for ERα (magenta) and PSD95 (green) in the CA1 lamina used for LTP experiments from prepubertal and adult females (deconvolved images; area of

overlap (white) identified in 3D). Arrows highlight double-labeled contacts ('2x synapses'). Line graph summarizes the density frequency distributions for ERα-ir co-localized with PSD95 as determined using Fluorescence Deconvolution Tomography. Adult females show a significant rightward skew towards high densities relative to prepubescent animals (2-way RM-ANOVA: $F_{19,342}=22.10$, $P=2.17\times10^{-48}$. N=10/group). Inset graph summarizes % higher density contacts (92+ on x-axis) (2-tailed unpaired t-test: **P=0.002 for pre- vs. post-puberty). (**c**) Representative image of ERβ (magenta) and PSD-95 (green) double-labeling. Synaptic ERβ density distribution curves were comparable in prepubertal and adult females ($F_{19,323}=0.16$, P=0.99. Prepub N=9, Adult N=10). Bar for **b-c**: 1 μm. Mean±SEM values shown. Statistics summarized in Supplementary Table 1 n.s., not significant.