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Cao G, Harris KM. Augmenting saturated LTP by broadly spaced episodes of theta-burst stimulation in hippocampal area CA1 of adult rats and mice. *J Neurophysiol* 112: 1916–1924, 2014. First published July 23, 2014; doi:10.1152/jn.00297.2014.—Hippocampal long-term potentiation (LTP) is a model system for studying cellular mechanisms of learning and memory. Recent interest in mechanisms underlying the advantage of spaced over massed learning has prompted investigation into the effects of distributed episodes of LTP induction. The amount of LTP induced in hippocampal area CA1 by one train (1T) of theta-burst stimulation (TBS) in young Sprague-Dawley rats was further enhanced by additional bouts of 1T given at 1-h intervals. However, in young Long-Evans (LE) rats, 1T did not initially saturate LTP. Instead, a stronger LTP induction paradigm using eight trains of TBS (8T) induced saturated LTP in hippocampal slices from both young and adult LE rats as well as adult mice. The saturated LTP induced by 8T could be augmented by another episode of 8T following an interval of at least 90 min. The success rate across animals and slices in augmenting LTP by an additional episode of 8T increased significantly with longer intervals between the first and last episodes, ranging from 0% at 30- and 60-min intervals to 13–66% at 90- to 180-min intervals to 90–100% at 240-min intervals. Augmentation above initially saturated LTP was blocked by the *N*-methyl-D-aspartate (NMDA) glutamate receptor antagonist D-2-amino-5-phosphonovaleric acid (D-APV). These findings suggest that the strength of induction and interval between episodes of TBS, as well as the strain and age of the animal, are important components in the augmentation of LTP.

spaced learning; metaplasticity; memory; synaptic plasticity

LONG-TERM POTENTIATION (LTP) is a persistent increase in synaptic strength, induced by brief high-frequency stimulation, that is widely accepted as a model for studying the cellular and molecular mechanisms of learning and memory (Bliss and Lomo 1973; Lynch et al. 1990). Some patterns of stimulation have no direct effect on synaptic strength but instead modulate the subsequent expression of long-term plasticity, a phenomenon known as metaplasticity (Abraham et al. 2001; Abraham and Bear 1996; Abraham and Tate 1997; Huang et al. 1992; Young and Nguyen 2005). Recently, there has been a resurgence of interest in the effect of spacing episodes of LTP induction as a model for understanding mechanisms of spaced learning (Lynch et al. 2013; Lynch and Gall 2013). Spaced learning produces longer memories than massed learning, and the efficacy of memory is dependent on the interval between repetitions (Ebbinghaus 1885; Fields 2005). Two paradigms, tetanic stimulation and theta-burst stimulation (TBS), have been most commonly used to investigate the metaplasticity of LTP. Initial experiments using tetanic stimulation in hippocampus in vivo or in slices from Wistar rats found that after LTP was saturated at one stimulus intensity, if sufficient time was allowed to pass and the stimulus intensity was reduced, later episodes of tetanus could be coaxed to produce more LTP (Frey et al. 1995). The TBS paradigm is more naturalistic than tetanus because it mimics firing patterns of hippocampal pyramidal cells in vivo (Bland 1986; Larson et al. 1986). One episode of TBS usually consists of 10 bursts at 5 Hz (theta) with each burst comprising 4 pulses at 100 Hz. In contrast to experiments using tetanic stimulation, when TBS was used to saturate initial LTP in young Sprague-Dawley (SD) rats, more potentiation could be produced at the same stimulus intensity an hour later (Kramar et al. 2012). Whether these findings reflect metaplastic regulation of LTP is an open question. One possibility is that the initial saturation of LTP triggers synaptic growth processes that are functionally silent and take time to manifest but once established provide a new substrate to augment the initially saturated LTP (Bell et al. 2014; Bourne and Harris 2011).

Conditions at the time when LTP is induced can affect whether subsequent stimulation will produce more LTP. For example, glutamate receptor activity is greatly enhanced by AMPAkines and the magnitude of initial LTP is much greater; thus subsequent induction of LTP is occluded in the presence of AMPAkines (Arai and Kessler 2007; Kramar et al. 2012). Less is known about the effects of different induction paradigms and delayed intervals on the capacity to produce additional LTP, and there is some ambiguity regarding the definitions of saturated, maximal, and asymptotic LTP. Prior work demonstrated that eight trains of TBS (8T) were required to induce maximal LTP in adult hippocampal area CA1 (Abraham and Huggett 1997), but it is not clear how long this maximal initial LTP lasts or whether it is saturated. In addition, age, circadian time when LTP is induced, and rodent strain can influence the magnitude and duration of synaptic potentiation (Bowden et al. 2012; Diana et al. 1994; Harris and Teyler 1983). Little is known about whether these conditions affect metaplasticity. Here we used 8T to induce LTP, which was considered to be initially saturated if repetition of 8T within a 5- to 30-min interval produced no additional potentiation. We compared the effects of massed versus spaced 8T episodes among two rat strains at two ages and one adult mouse strain. The results show that age, strain, spacing interval, and the magnitude of initial LTP all play important roles in metaplasticity and subsequent augmentation of LTP in hippocampal area CA1.

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MATERIALS AND METHODS

Slice preparation. Procedures were approved by the University of Texas at Austin Institutional Animal Care and Use Committee and complied with all National Institutes of Health requirements for the humane care and use of laboratory animals. Hippocampal slices were rapidly prepared from young male SD rats (30–42 days old), young Long-Evans (LE) (30–42 days old) and adult LE (50–65 days old) rats, and adult C57BL/6J mice (7–9 wk old). Animals were anesthetized with isoflurane and then decapitated with a guillotine. The brain was removed from the cranium, and the left hippocampus was dissected out and rinsed with room temperature artificial cerebrospinal fluid (aCSF) containing (in mM) 117 NaCl, 5.3 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, and 10 glucose, pH 7.4, and bubbled with 95% O_2-5% CO_2. Four slices (400 μm thick) from the dorsal-lateral part of the hippocampus were cut at 70° transverse to the long axis on a tissue chopper (Stoelting, Wood Dale, IL) and transferred in oxygenated aCSF to the supporting nets of interface chambers in the Synchroslice system (Lohmann Research Equipment, Castrop-Rauxel, Germany). The entire dissection and slice preparation took <5 min, which is crucial timing for enduring LTP that lasts >3 h (Reymann and Frey 2007). This rapid dissection, together with the interface chamber design, provides for the maintenance of glial and neuronal integrity and high-quality ultrastructure during long acute slice experiments (Bourne et al. 2007a; Bourne and Harris 2012; Harris and Teyler 1984; Jensen and Harris 1989; Takano et al. 2014).

Electrophysiology. Hippocampal slices were placed on a net at the liquid-gas interface between 32–32.5°C aCSF and humidified 95% O_2-5% CO_2 atmosphere bubbled through 35–36°C distilled water. After 3 h of incubation, the stimulating and recording electrodes were positioned 400 μm apart in the middle of hippocampal CA1 stratum radiatum with the stimulating electrode on the CA3 side. Stimuli consisted of 200-μs biphasic current pulses, lasting 100 μs each for positive and negative components of the stimulus. Test pulses (100–250 μA) were given at 1 pulse per 2.5 min unless stated otherwise, and field excitatory postsynaptic potentials (fEPSPs) were recorded. Right after the TBS was applied, fEPSPs were recorded every 10 s for 1 min to examine whether short-term, post-TBS potentiation occurred. Also, when two episodes of TBS were given with a 5-min interval to saturate initial LTP (see RESULTS for details), the fEPSPs were recorded every 30 s for 4 min after 1-min recordings at every 10 s to test for short-term, post-TBS potentiation. The initial fEPSP slope was ~40% of the maximal fEPSP slope based on the input/output curve for each slice. LTP was induced by one train (1T) of TBS or eight trains of TBS with 30-s intervals (8T) as indicated in RESULTS. Each train of TBS contained 10 bursts at 5 Hz, and each burst contained 4 pulses at 100 Hz. For the metaplasticity experiments, additional episodes of high-frequency stimulation were applied at the intervals indicated in RESULTS, and fEPSPs evoked by test pulses were recorded thereafter. *N*-methyl-D-aspartate glutamate receptor (NMDAR) activation was blocked by adding 4 μl of D,L-2-amino-5-phosphonovaleric acid (D,L-APV; 25 mM) to the 1 ml of aCSF in the interface recording chamber, which achieved an effective concentration of 50 μM D-APV.

Data analysis. The delivery of presynaptic stimulation and the acquisition and analysis of fEPSPs were performed with Synchro-Brain software (Lohmann Research Equipment). The initial maximum slope was measured over a 0.4-ms time frame that was held constant for all recordings in each slice. To calculate the magnitude of LTP, the average fEPSP slopes during the last 25 min of baseline recordings before the delivery of the first TBS were computed and then compared to the average values during the last 25 min of each session following delivery of TBS. Then values across slices (means ± SE) were presented as times baseline, where 1 indicated no change in the fEPSP slope relative to the pre-TBS baseline. We used times baseline (instead of %) here so that direct comparisons could be made more easily to an earlier study (Kramar et al. 2012).

The STATISTICA software package (StatSoft, Tulsa, OK) was used for statistical analysis. When levels of LTP were compared across ages and strains, an overall ANOVA was done first, followed by post hoc Tukey's test (e.g., Fig. 1 and related text regarding initial levels of LTP). When only two groups of data were compared, Student's *t*-test was used. The production of additional LTP required meeting two criteria: *1*) there was a significant increase ($P < 0.05$) in fEPSP slope after additional episodes of TBS calculated for the last 25 min of the recording time between episodes of TBS and *2*) the average additional increase in the fEPSP slope was at least 10%. Pearson's χ^2-tests were used to test whether the success rate of producing additional LTP is dependent on the length of intervals between the first and last episodes of 8T.

RESULTS

Strain-specific differences in augmentation of LTP. Rats are known to exhibit various strain-specific differences in long-term synaptic plasticity (Bowden et al. 2012; Manahan-Vaughan 2000; Manahan-Vaughan and Schwegler 2011). Hence, we first tested whether hippocampal slices from LE and SD strains of rats respond differently to repeated episodes of LTP induction. Using the same pattern of TBS stimulation as Kramar et al. (2012), we delivered four episodes of the 1T paradigm at 1-h intervals to stratum radiatum in area CA1 of hippocampal slices (Fig. 1). In young SD rats, the first 1T induced LTP and the second and third episodes of 1T each produced significantly more potentiation, while the fourth 1T produced no further significant increase (Fig. 1*A*), which is consistent with previous reports (Kramar et al. 2012). However, hippocampal slices from neither young (Fig. 1*B*) nor adult (Fig. 1*C*) LE rats exhibited significantly more potentiation following multiple episodes of 1T at 1-h delays.

Next, we tested whether the number of trains within a single episode of TBS played a role in age, strain, or species differences in the augmentation of LTP. Others have shown that eight trains of TBS delivered at 30-s intervals (8T) are needed to induce maximal LTP in hippocampal area CA1 of adult SD rats (Abraham and Huggett 1997). Prior work in young SD rats demonstrated that a 30-min interval was not sufficient time for a second episode of 1T to produce additional LTP, suggesting that 1T saturated LTP at this age (Kramar et al. 2012). To test this saturation hypothesis, we delivered 8T at 30, 60, or 120 min after the first episode of 1T. In young SD rats, the potentiation produced by 1T was not statistically different from that achieved after an episode of 8T delivered 30 min later (Fig. 2*A*). Interestingly, unlike 1T, additional episodes of 8T delivered 60 or 120 min later also did not allow more LTP, suggesting that the duration of saturation is longer after 8T than after 1T in young SD rats (compare Fig. 1*A* and Fig. 2*A*). In contrast, both young (Fig. 2*B*) and adult (Fig. 2*C*) LE rat and adult C57BL/6 mouse (Fig. 2*D*) hippocampal slices showed significantly more LTP when 8T was delivered 30 min after 1T; however, additional episodes of 8T delivered at 60 or 120 min later also did not produce more LTP, as in young SD rats (Fig. 2*E*). These results indicated that, unlike young SD rats, 8T was required initially to saturate LTP in young and adult LE rats and in adult mice.

Results from both sets of experiments were pooled to discern whether there were strain, age, or species differences in the absolute level of initial LTP achieved that might explain the clear saturation by 1T in young SD rats and the lack of

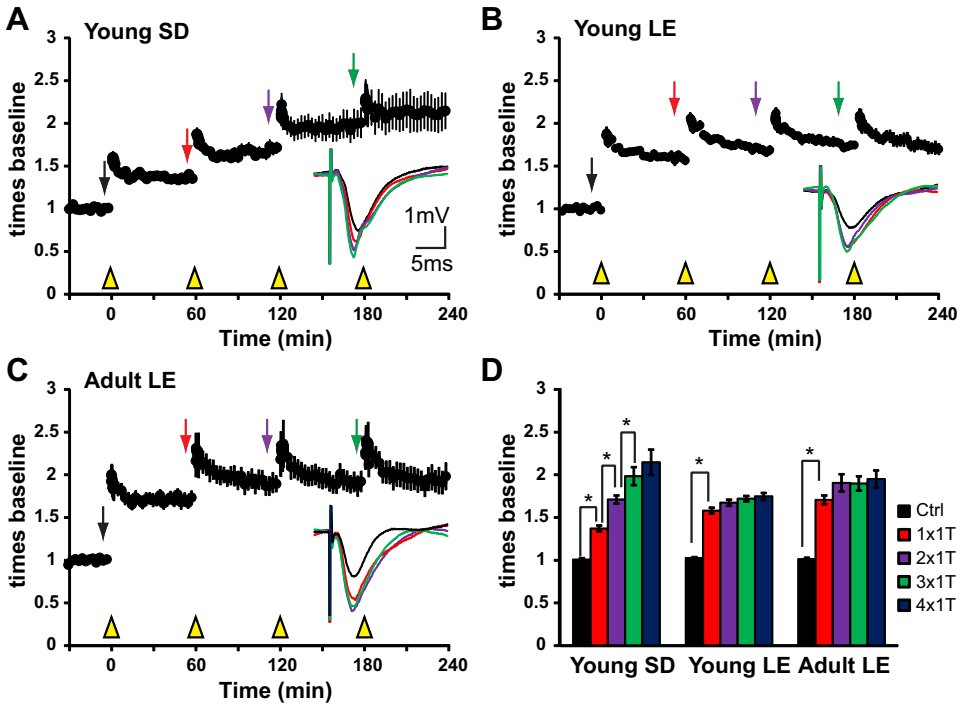

Fig. 1. Strain-specific differences between young Sprague-Dawley (SD) and young or adult Long-Evans (LE) rats in augmentation of long-term potentiation (LTP) by the 1T paradigm. *A*: in hippocampal slices from young SD rats ($n = 10$) the first episode of theta-burst stimulation (TBS) induced LTP (1.37 ± 0.02 times of baseline, mean ± SE) and the second and third episodes of 1T each produced significantly more potentiation [1.71 ± 0.04 ($P < 0.01$) and 1.98 ± 0.15 ($P < 0.05$), respectively], but the fourth 1T did not produce significantly more potentiation (2.14 ± 0.31, $P = 0.38$). *B*: in slices from young LE rats ($n = 11$) the first episode of TBS induced LTP (1.58 ± 0.04), with no further significant increases from the second, third, and fourth episodes. *C*: similarly, in slices from adult LE rats ($n = 9$) the first episode of TBS induced LTP (1.70 ± 0.05), and the second, third, and fourth episodes did not produce significantly more potentiation. *D*: summary graph shows where potentiation was >10% and differed significantly (*) from baseline stimulation or sequential episodes of 1T, as computed over the last 25 min of recording between each 1T. Yellow triangles, 1 episode of TBS; times baseline(*y*-axis), fold changes in the initial maximal slope of the field excitatory postsynaptic potential (fEPSP) relative to the 30-min baseline. Traces are representative recordings at different times indicated by arrows color-matched to the summary data.

saturation in young or adult LE rats or mice (Fig. 3). Indeed, young SD rats produced significantly less LTP than young LE rats ($P < 0.01$) and young LE rats produced significantly less LTP than adult LE rats ($P < 0.05$), but there were no differences between adult LE rats and adult mice ($P = 0.44$). As expected from this analysis, both adult LE rats and mice showed more initial LTP than young SD rats ($P < 0.01$). Thus the relatively weak induction paradigm of 1T initially saturated LTP in young SD rats, but a stronger induction paradigm (e.g., 8T) was needed initially to saturate LTP in young or adult LE rats or mice. These findings suggest that the saturation of LTP depends on the underlying response capacity of these fundamentally different nervous systems.

Testing initial saturation of LTP by 8T in adult LE rats. Multiple episodes of 8T were given to test whether LTP was initially saturated in hippocampal slices from adult LE rats. One episode of 8T induced stable LTP such that the potentiation measured at 5 min after TBS (2.04 ± 0.07) was comparable to that measured at 120 min after the 8T (2.09 ± 0.09, $P = 0.59$; Fig. 4*A*). To test for initial saturation, two episodes of 8T were delivered at a 5-min interval. The magnitude of potentiation after the first episode (2.17 ± 0.17) was comparable to that measured at 120 min after the second 8T (2.04 ± 0.14, $P = 0.55$; Fig. 4*B*). For all subsequent experiments, two episodes of 8T were delivered at a 5-min interval to confirm that LTP was initially saturated. Then additional episodes of 8T were delivered at later times to determine whether LTP remained saturated. Not surprisingly, a third episode of 8T delivered 30 min (Fig. 4*C*) or 60 min (Fig. 4*D*) later did not produce more potentiation. Thus one episode of 8T saturated initial LTP for at least 60 min in hippocampal slices from adult LE rats.

Augmentation of LTP is dependent on time interval between episodes of TBS. So far, we have demonstrated that 8T is required to saturate initial LTP in both young and adult LE rats and adult mice. In addition, LE rats of both ages and adult mice did not produce more LTP after repeated episodes of 1T. Therefore, we wanted to test whether 8T had permanently saturated LTP or longer delays might reveal a new capacity to augment LTP. When the interval between the first and last episodes of 8T was 90, 120, or 150 min, <50% of slices showed more LTP. However, when the interval was increased to 180 min, only 30% of slices did not produce additional potentiation (Fig. 5*A*) and 70% showed augmentation of LTP (Fig. 5*B*). When the interval was increased to 240 min, 92% of all slices from adult LE rats showed additional LTP (Fig. 5*C*) and 100% of adult mice showed more LTP (Fig. 5*D*). Interestingly, the magnitude of LTP augmentation produced after a 240-min interval was significantly greater in adult mice (34.3% ± 2.7%) than in adult LE rats (15.2% ± 0.6%, $P < 0.002$).

The NMDAR plays a crucial role in the initial induction of LTP at hippocampal CA3→CA1 synapses, so we tested whether the augmentation of LTP was also NMDAR depen-

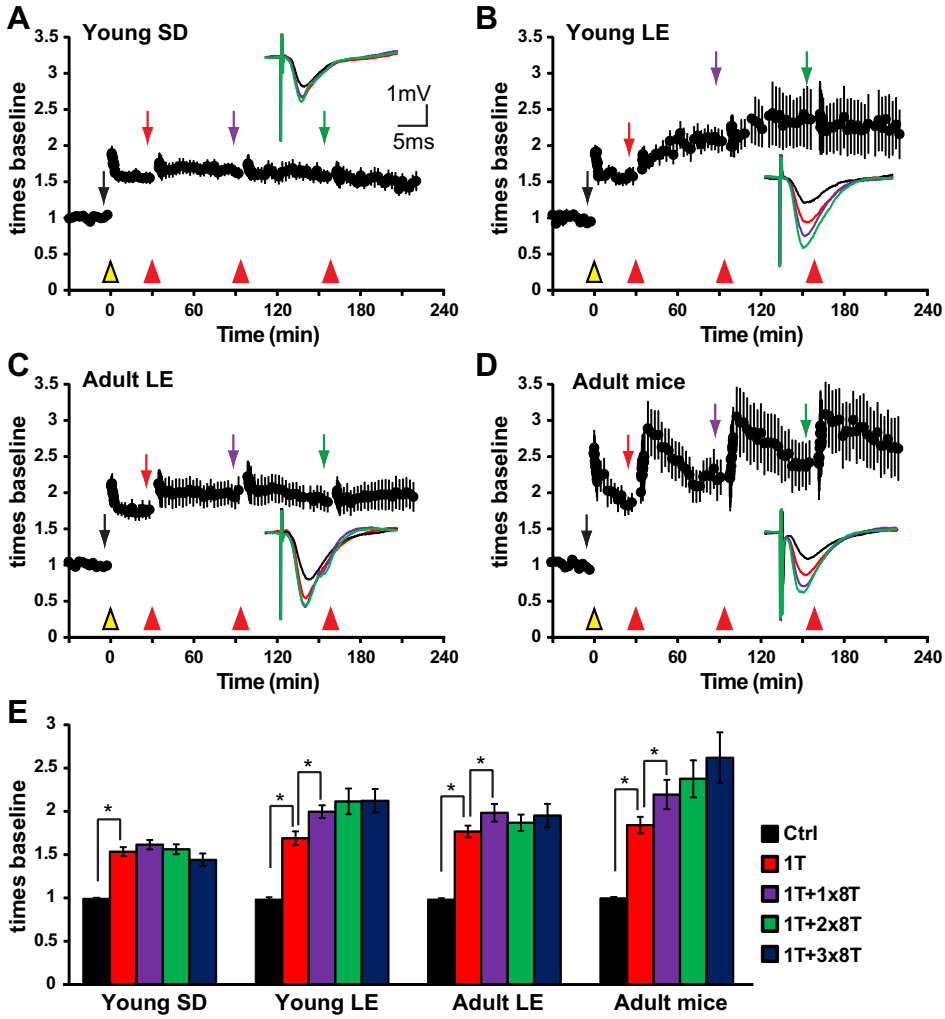

Fig. 2. One episode of 1T produces saturated LTP in young SD rats but not LE rats and mice. *A*: in young SD rats ($n = 12$) LTP produced by 1T remained at the same level after 8T at 30, 60, or 120 min. *B*: in young LE rats ($n = 6$) the level of potentiation following 1T (1.69 ± 0.09) increased after 1 episode of 8T (2.00 ± 0.07, $P < 0.01$) and remained saturated after 8T episodes delivered at 2 additional 60-min intervals ($P > 0.05$). *C*: in adult LE rats ($n = 13$), the potentiation following 1T (1.76 ± 0.11) was also increased after 1 episode of 8T (1.98 ± 0.24, $P < 0.05$) but not after subsequent 8T episodes ($P > 0.05$). *D*: in adult mice ($n = 8$) the potentiation following 1T (1.84 ± 0.15) was also further increased after 1 episode of 8T (2.19 ± 0.47, $P < 0.01$) but not after subsequent 8T episodes ($P > 0.05$). *E*: summary graph shows where potentiation was >10% and differed significantly from prior episodes of 1T or 8T (*$P < 0.05$). *A–D*: yellow triangles, 1T; red triangles, 8T. Traces are representative recordings at different times indicated by arrows color-matched to the summary graphs.

dent, using the NMDAR antagonist D-APV. D-APV was added to the recording chamber 1 h before delivery of the third episode of 8T at 240 min. This application of D-APV had no effect on the established LTP, but it completely blocked the production of additional LTP (Fig. 5*E*), demonstrating a requirement for NMDAR activation.

Finally, we assessed whether the probability of producing additional LTP was significantly greater as the interval between 8T episodes was increased (Fig. 5*F*). Overall, there was a significant association between the expression of additional LTP and the time interval in adult LE rats (Pearson's χ^2-test, $P < 0.05$). There were no significant differences between the 30- and 60-min intervals; the 90-, 120-, and 150-min intervals; or the 180- and 240-min intervals (χ^2-test, $P > 0.05$). However, when the success rates were compared between these three groups of intervals there was a significant increase in success with more time (Fig. 5*F*; $P < 0.05$). Thus augmentation of LTP depends on the time interval between episodes of 8T.

Baseline response and initial saturation across intervals. Field recordings are the only measure that can be held for sufficient time to test whether LTP was saturated and when it

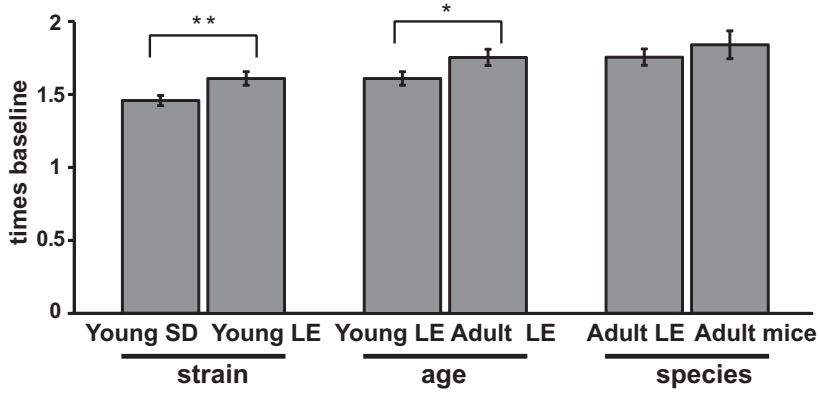

Fig. 3. Strain, age, and species differences in the magnitude of LTP induced by 1T. The magnitude of LTP induced by 1T was 1.46 ± 0.03 in young SD rats ($n = 16$), which was significantly lower than in young LE rats (1.61 ± 0.05, $n = 13$; **$P < 0.001$). This magnitude in young LE rats was significantly less that that achieved in adult LE rats (1.76 ± 0.06, $n = 21$, *$P < 0.05$), which did not differ significantly from adult mice (1.84 ± 0.10, $n = 8$, $P = 0.44$).

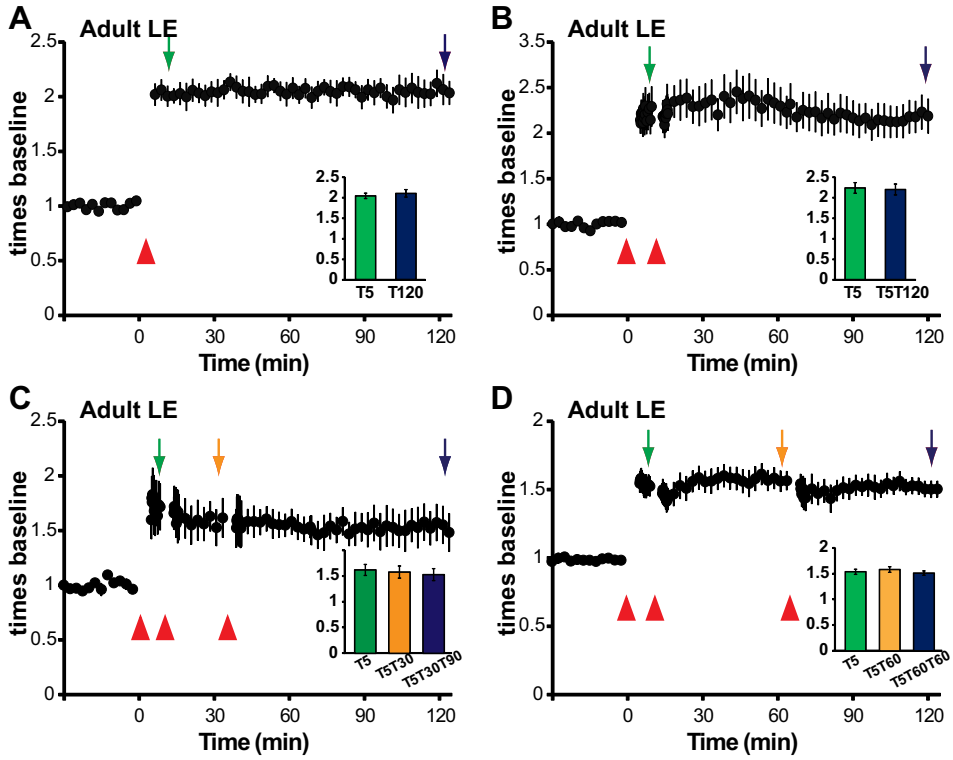

Fig. 4. Initially saturated and stable LTP induced by 1 episode of 8T in adult LE rats. *A*: 1 episode of 8T (red triangle) induced stable LTP as determined by fEPSP slopes at 5 min (green arrow and bar) and 120 min (blue arrow and bar), showing no significant difference ($n = 10$, $P = 0.31$). *B*: the fEPSP slope measured after 1 episode of 8T (green arrow and bar) showed no additional potentiation after a second 8T episode measured 120 min later (blue arrow and bar, $n = 20$, $P = 0.84$). *C*: when a third episode of 8T was given 30 min after the first two, no additional potentiation was produced ($n = 6$, $P = 0.85$). *D*: similarly, when the third episode of 8T was delivered 60 min after the first 2 episodes of 8T, no additional potentiation was produced ($n = 8$, $P = 0.59$).

might be augmented. The absolute magnitude of the fEPSP response and degree of plasticity can vary from slice to slice, so great care was taken to establish a baseline response that was positioned comparably along the input/output response curve at the beginning of each experiment, namely, at ~40% of maximal response capacity for each slice. Among the three time interval groups described above in adult LE rats (Fig. 5*F*), there were no significant differences in the magnitude of the baseline fEPSP slopes (Fig. 6*A*). In addition, the level of initial LTP saturation did not differ significantly among these intervals (Fig. 6*B*). These results suggest that differences in the absolute magnitude of baseline response or level of initial saturation were not responsible for the interval-dependent augmentation of LTP in adult LE rat hippocampus.

DISCUSSION

These findings suggest that both the initial saturation and later augmentation of LTP depend on many factors, including the strength of the induction paradigm, animal strain and age, and the interval between episodes of TBS. We defined augmentation of LTP as being both statistically significant and >10% above the initially saturated level of LTP. A relatively weak induction paradigm involving a single TBS train saturated initial LTP for 1 h in young SD rats, and then two subsequent episodes of 1T at 1-h intervals produced more LTP. In contrast, a stronger LTP induction paradigm involving 8T was required to saturate LTP in both young and adult LE rats and adult C57BL/6J mice. Under these conditions, later augmentation of LTP was both time and NMDAR dependent, and at an interval of 240 min there was a 90–100% success rate in adult LE rats and mice.

In the hippocampus from Wistar, LE, SD, and other rat strains and most wild-type mice, LTP is readily expressed and shows similar changes in synaptic receptor function and downstream signaling. Therefore, age, strain, and species differences are often overlooked. Recent work shows, however, that the magnitude of LTP produced in the hippocampal dentate gyrus in vivo is substantially greater in adult LE than in adult SD rats (Bowden et al. 2012), an effect that is amplified during the dark cycle (Bowden et al. 2012; Harris and Teyler 1983). The age of the animal can have a profound influence on LTP, which can also be confounded by induction paradigm. In LE rats, the onset age of enduring LTP from 8T is postnatal day 12 while tetanic stimulation first produces enduring LTP at postnatal day 15 (Cao and Harris 2012; Harris and Teyler 1983; Jackson et al. 1993). Furthermore, the magnitude of LTP induced by either paradigm increases substantially with maturation. Age and strain also influence the saturation of LTP. In young SD rats (30–42 days) we confirmed that 1T is sufficient to saturate initial LTP (see also Kramar et al. 2012), whereas in hippocampus from young or adult LE rats or adult mice 1T does not saturate LTP, as demonstrated by production of more LTP when 8T was delivered 30 min later. Finally, augmentation of LTP was differentially modulated by these factors. In young SD rats but not young or adult LE rats episodes of 1T delivered at 1-h intervals augmented LTP, and the magnitude of LTP augmentation by 8T was greater in adult mice than in adult LE rats. Thus induction paradigm, strain, and age all can influence both the magnitude of initial saturation and the level of LTP augmentation. It remains to be determined whether these influences reflect diverse underlying mechanisms between rats of different strains and ages and mice, such as fewer synapses in young rats or adult mice (Kirov et al. 2004; Routh et al. 2009), or different levels of prior, experience-based LTP or metaplasticity (Barnes et al. 1994; Habib et al. 2013; Huang et al. 1992; Moser et al. 1998; Takeuchi et al. 2014).

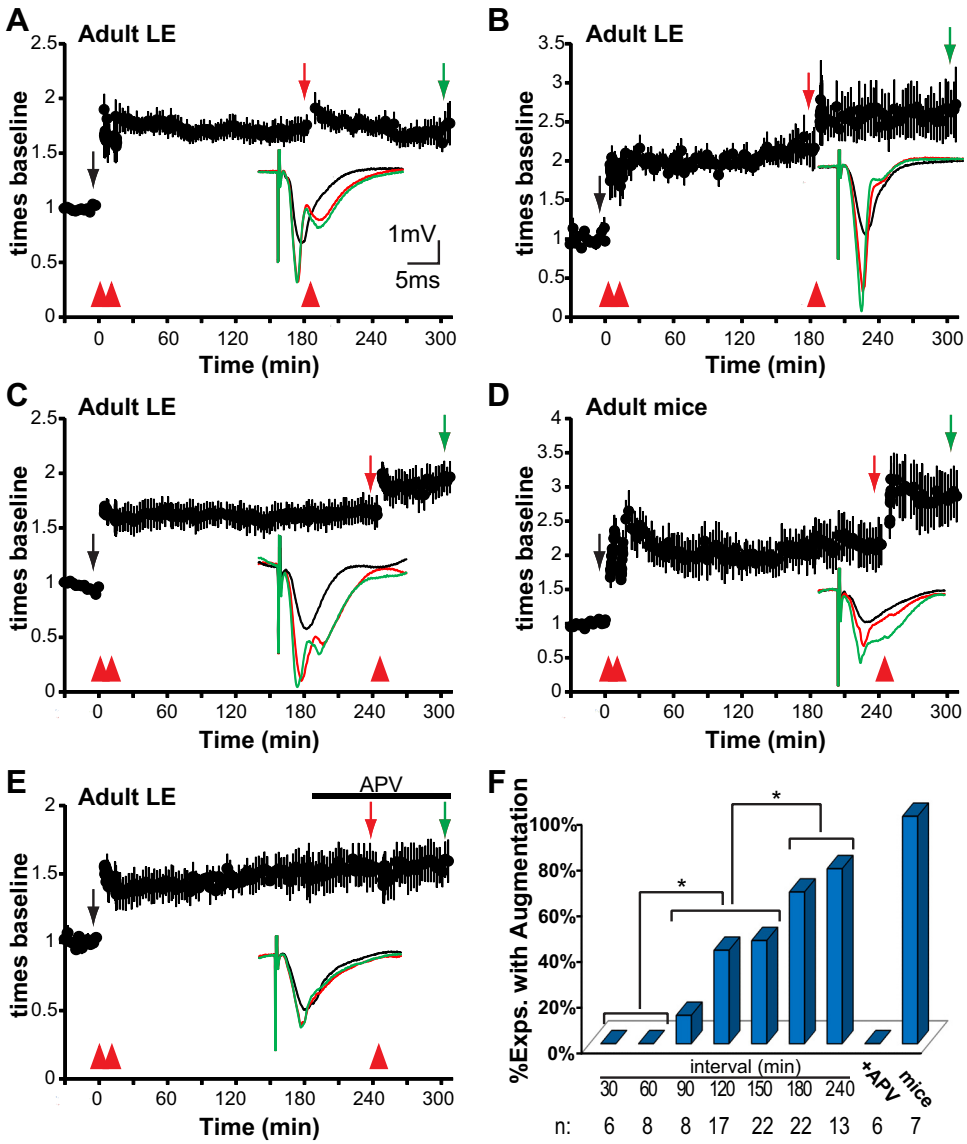

Fig. 5. Augmentation of LTP at longer intervals between second and third 8T episodes. At a 180-min interval, some slices show no change ($n = 7$, $P = 0.80$, *A*), while others show augmented LTP (*B*) when comparing the level of potentiation before (2.12 ± 0.25) and after the last 8T (2.57 ± 0.29; $n = 14$, $P < 0.05$). *C*: at a 240-min interval, 12 of 13 slices show additional LTP when comparing potentiation before (1.64 ± 0.15) and after the last 8T (1.85 ± 0.64; $n = 13$, $P < 0.05$). *D*: in adult mice, all slices showed additional LTP at the 240-min interval when comparing potentiation before (2.14 ± 0.62) and after the last 8T (2.91 ± 0.91; $n = 7$, $P < 0.05$). *E*: blocking the NMDA receptor (NMDAR) with D-2-amino-5-phosphonovaleric acid (D-APV; 50 μM) prevented the production of additional LTP at 240 min ($n = 6$). *F*: % of slices showing additional LTP at different intervals: 0% at 30 and 60 min, 13% at 90 min, 35% at 120 min, 36% at 150 min, 66% at 180 min, and 92% at 240 min. *Significant differences by χ^2 analyses as discussed in the text ($P < 0.05$). Red triangle, 1 episode of 8T. Representative recordings at different time points are indicated by color-matched arrows in *A*–*E*: control (black), LTP before (red) and after (green) the last 8T. n = total no. of slices tested.

Although LTP was initially saturated by 1T or 8T in young SD rats, 60 min was sufficient time for subsequent 1T, but not 8T, to produce augmentation of LTP. These findings suggest that the stronger 8T stimulation paradigm might saturate signaling or structural mechanisms needed for the augmentation of LTP. This saturation is reminiscent of suppression of LTP induction when the number of trains of TBS in adult SD rats is >16; indeed, 34 trains caused depression instead of potentiation (Abraham and Huggett 1997). Similarly, multiple episodes of 8T did not produce augmentation of LTP in adult LE rats during four episodes delivered at 0, 5, 60, and 120 min (for a total of 120 min between the first and last episode), whereas three episodes at 0, 5, and 120 min produced augmentation of LTP in ~50% of experiments. These findings suggest that the close spacing of 8T either exhausts resources needed for augmentation of LTP or extends the refractory period induced by the initial saturation with 8T. Thus initial stimulation appears to be critical in determining the time needed to prepare synapses for later augmentation of LTP.

The intensity of the second episode following induction of LTP appears to influence whether LTP will be augmented. Barr and colleagues (1995) found that increasing the stimulation intensity during a second TBS episode depressed prior LTP. Frey and colleagues (1995) found that it was necessary to reduce the stimulation intensity in order to produce more LTP, even when the two episodes of tetanic stimulation were separated by a 4-h interval. In contrast, we held stimulation intensity constant across episodes of TBS and found that LTP was augmented with increasing reliability as the interval between the first and last episodes increased from 1.5 to 4 h. These discrepancies could be attributed to the differences in animal strain, slice conditions, or LTP induction protocols.

Many mechanisms could account for the long delay required to augment LTP after the 8T paradigm that initially saturated LTP in adult LE rats and mice. An attractive hypothesis is that sufficient time must pass to achieve the changes in the composition or structure of dendritic spines and synapses that are required to respond to subsequent plasticity-inducing stimuli (Bourne and Harris 2007). Recent work suggests that dendritic spines have different thresholds for LTP induction, and more spines are recruited in young SD rats with each episode of 1T at 1-h intervals, as demonstrated by their enhanced actin

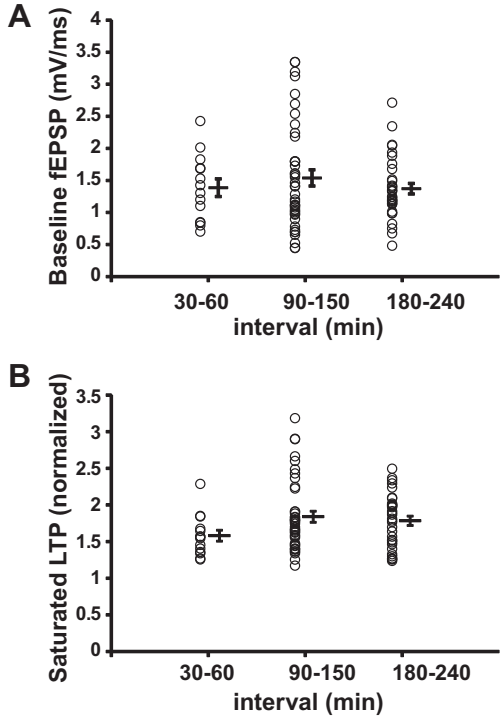

Fig. 6. Baseline fEPSP slope and magnitude of initial LTP saturation did not predict augmentation of LTP. *A*: baseline fEPSP slopes are plotted (open circles) for each experiment and grouped across the 3 sets of time intervals established in Fig. 4*F*. Average baseline fEPSPs (beam with error bars, mean ± SE) were not significantly different among the 3 groups (ANOVA, $P = 0.52$). *B*: magnitude of saturated LTP was also plotted for each experiment (open circles) and grouped across the 3 sets of time intervals. The average magnitude of saturated LTP was not significantly different among the 3 groups (ANOVA, $P = 0.14$).

polymerization status (Kramar et al. 2012). The first saturating TBS potentiates a subpopulation of dendritic spines and also primes neighboring spines via the spread or production of key signaling molecules so that neighboring synapses can be potentiated by subsequent LTP induction stimuli (Harvey and Svoboda 2007; Kramar et al. 2012). This time-dependent process may also involve calpain-associated breakdown and synthesis of suprachiasmatic nucleus circadian oscillatory protein (SCOP), which negatively regulates the extracellular signal-regulated kinase (ERK) and alters the actin cytoskeleton of dendritic spines during LTP (Wang et al. 2014). The altered cytoskeleton in neighboring spines may serve to enhance transport of key molecules from the dendritic shaft as part of the process needed to prepare them for potentiation by subsequent TBS. The synaptic adhesion receptors belonging to the β1 integrin family may also play an important role in the delay required for LTP augmentation, as previous findings showed that β1 integrins were critical for LTP consolidation and the activation of β1 integrins by TBS was brief and was followed by a refractory period of ~45 min, during which they did not respond to another episode of TBS (Babayan et al. 2012).

Enduring LTP also engages local protein synthesis at activated synapses (Bourne et al. 2007b; Bramham 2008; Tom Dieck et al. 2014). The mRNAs translated locally at potentiated synapses could come from at least three sources: mRNAs previously deposited in the spines, mRNAs that are transported into spines from the dendrites, and replenishment of these local stores of mRNA with transport of new mRNA from the soma. Studies on the immediate-early gene activity-regulated cytoskeleton-associated protein (*Arc*) demonstrated that local translation of *Arc* at synapses plays an essential role in the maintenance of LTP and in the consolidation of long-term memory (Guzowski et al. 2000). High-frequency stimulation triggers the transcription of the *Arc* gene in the soma, and newly synthesized *Arc* mRNA is transported to activated synapses over the next 1–2 h (Steward et al. 1998; Steward and Worley 2001, 2002; Yilmaz-Rastoder et al. 2011). The *Arc*-dependent stabilization of newly polymerized F-actin in activated spines is essential for LTP consolidation (Bramham 2008; Messaoudi et al. 2007). Thus the extended delay between the first and last episodes of TBS could involve the time required for newly transcribed mRNAs, such as *Arc*, to be transported from the cell body to dendrites and into spines with enhanced actin cytoskeleton, where they could provide the priming needed to respond to subsequent TBS.

Recently we reported that induction of LTP by 8T results in the elimination of some small dendritic spines with a commensurate enlargement of synapses on the remaining spines of all sizes by 2 h (Bourne and Harris 2011). Presynaptic boutons were lost at the same frequency as small spines (Bourne et al. 2013). The synapse enlargement entailed conversion of nascent zones at the edges of mature synapses to active zones (Bell et al. 2014; Spacek and Harris 1998). The postsynaptic density (PSD) at nascent zones is continuous with the PSD of the active zone, but no presynaptic vesicles congregate at nascent zones. Nascent zones were found at ~35–55% of synapses and occupied ~20% of the PSD of those synapses. By 30 min after induction of LTP with 8T, recruitment of presynaptic vesicles converted many of the existing nascent zones to active zones. However, by 2 h after induction of LTP, the nascent zones were enlarged and new nascent zones were added. The vesicle docking sites were on average >200 nm away from nascent zones; hence, glutamate concentrations at nascent zones are likely to be too low to activate AMPA receptors in them, which would make them functionally silent (Franks et al. 2002, 2003). Thus the silence of nascent zones during the interval would account for the stability of the originally saturated LTP, and the timing of nascent zone growth could prepare synapses for augmentation of LTP, which is conceptually similar to the partially silent synapse hypothesis (Lisman and Raghavachari 2006).

Hippocampal LTP provides a good model for studying learning and memory. Both LTP and learning vary greatly depending on the context, intensity, and duration of an experience. The effects of a high-intensity and long learning session could be quite different from multiple shorter sessions in regard to the magnitude of LTP induced and synapses involved. Spaced or distributed learning is known to be more effective than massed learning. Our findings show that saturation of initial LTP varies by induction protocol, age, strain, and species. The augmentation of LTP depends on the spacing between TBS episodes. In adult LE rats and mice, 90–100% of slices show robust augmentation when given a 4-h delay, while augmentation is rare when the delay between episodes is <2 h. Both learning and LTP can become impaired in old age, but distributed training can counteract these effects, and it will be interesting to learn whether the augmentation of LTP is similarly modulated in old age (Foster 2012; Lynch 1998; Lynch et al. 2006).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: G.C. and K.M.H. conception and design of research; G.C. performed experiments; G.C. analyzed data; G.C. and K.M.H. interpreted results of experiments; G.C. prepared figures; G.C. and K.M.H. drafted manuscript; G.C. and K.M.H. edited and revised manuscript; G.C. and K.M.H. approved final version of manuscript.

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